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⑤④ **Vascular endothelial cell growth factor C subunit.**

⑤⑦ Vascular endothelial cell growth factor C subunit DNA is prepared by polymerase chain reaction techniques. The DNA encodes a protein that may exist as either a heterodimer or homodimer. The protein is a mammalian vascular endothelial cell mitogen and as such is useful for the promotion of vascular development and repair. This unique growth factor is also useful in the promotion of tissue repair.

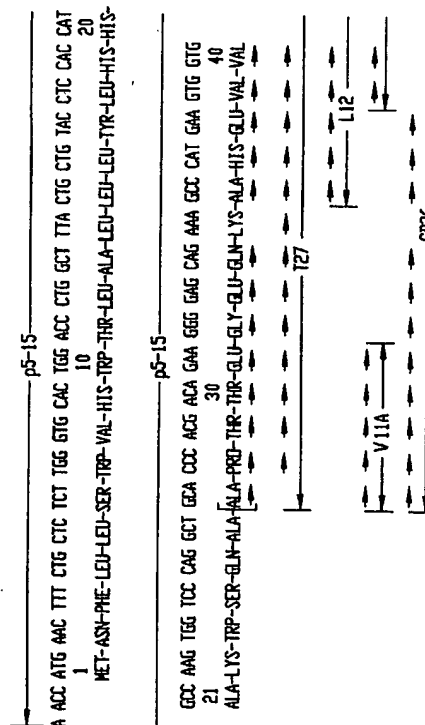
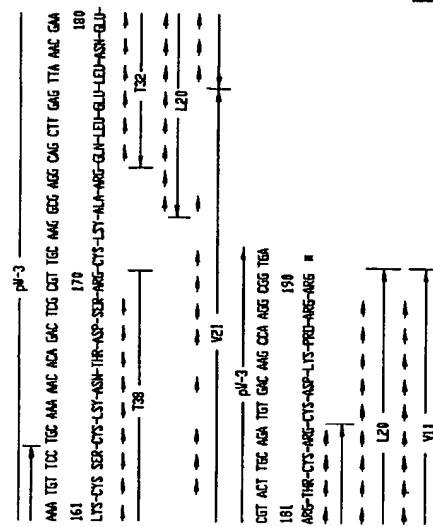
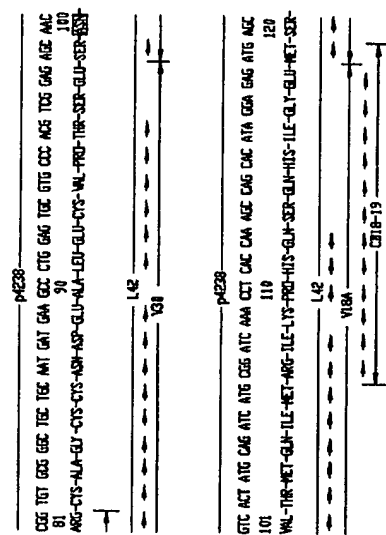
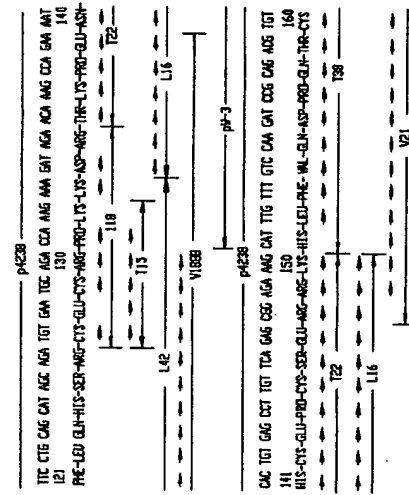
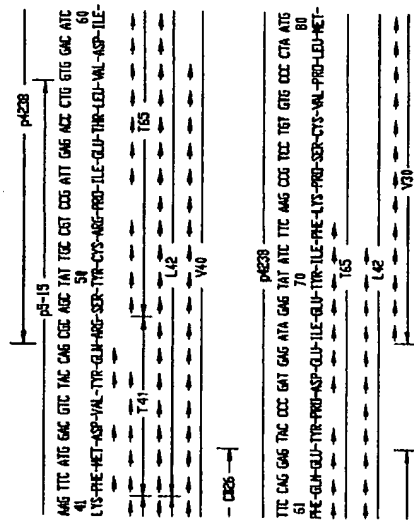


FIG.1A

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BRIEF DESCRIPTION OF THE DRAWING

Figure 1. Full length amino acid residue protein translation product and its cDNA coding sequence for VEGF AA subunit A plus polypeptide cleavage products used to determine the amino acid sequence.

5 Figure 2. Full length amino acid residue protein translation product and its cDNA coding sequence for VEGF AB subunit A plus polypeptide cleavage products used to determine the amino acid sequence.

Figure 3. Full length amino acid residue protein translation product and its cDNA coding sequence for VEGF AB subunit B plus polypeptide cleavage products used to determine the amino acid sequence.

10 Figure 4. Full cDNA coding sequence and full length amino acid residue protein translation product for VEGF A 146 amino acid residue subunit SEQ ID NOS:23 & 33.

Figure 5. Full cDNA coding sequence and full length amino acid residue protein translation product for VEGF A 190 amino acid residue subunit SEQ ID NOS:30 & 31.

Figure 6. Full cDNA coding sequence and full length amino acid residue protein translation product for VEGF A 214 amino acid residue subunit SEQ ID NOS:34 & 35.

15 Figure 7. Full cDNA coding sequence and full length amino acid residue protein translation product for VEGF B 138 amino acid residue subunit SEQ ID NOS:36 & 37.

Figure 8. Full cDNA coding sequence and full length amino acid residue protein translation product for VEGF B 158 amino acid residue subunit SEQ ID NOS:38 & 39.

20 Figure 9. Full cDNA coding sequence and full length amino acid residue protein translation product for VEGF C 154 amino acid residue subunit SEQ ID NOS:40 & 41.

BACKGROUND OF THE INVENTION

25 A new class of cell-derived dimeric mitogens with apparently restricted specificity for vascular endothelial cells has recently been identified and generally designated vascular endothelial growth factors (VEGFs). The mitogen has been purified from: conditioned growth media of rat glioma cells, [Conn *et al.*, Proc. Natl. Acad. Sci. USA **87**: 1323-1327 (1990)]; conditioned growth media of bovine pituitary folliculo stellate cells [Ferrara and Henzel, Biochem. Biophys. Res. Comm. **161**: 851-858 (1989) and Gospodarowicz *et al.*, Proc. Natl. Acad. Sci. USA **86**: 7311-7315 (1989)]. An endothelial cell growth factor isolated from mouse neuroblastoma cell line NB41 with an unreduced molecular mass of 43-51 kDa and a reduced mass of 23-29 kDa has been described by Levy *et al.*, Growth Factors **2**: 9-19 (1989). Connolly *et al.* (J. Biol. Chem. **264**: 20017-20024 [1989]; J. Clin. Invest. **84**: 1470-1478 [1989]) describe a human vascular permeability factor that stimulates vascular endothelial cells to divide *in vitro* and promotes the growth of new blood vessels when administered into healing rabbit bone grafts or rat corneas. An endothelial cell growth factor has been purified from the conditioned medium of the AtT-20 pituitary cell line by Plouet *et al.*, EMBO Journal **8**: 3801-3806 (1989). The growth factor was characterized as a heterodimer composed of subunits with molecular mass of 23 kDa. Leung *et al.* (Science **246**: 1306-1309 [1989]), Keck *et al.* (Science **246**: 1309-1312 [1989]) and Conn *et al.* (Proc. Natl. Acad. Sci. USA **87**: 2628-2632 [1990]) have described cDNAs which encode VEGF A which is homologous to the A and B chains of platelet-derived growth factor. Vascular endothelial growth factor I (VEGF I, VEGF AA) is a homodimer with an apparent molecular mass of 46 kDa, with each subunit having an apparent molecular mass of 23 kDa. VEGF I has distinct structural similarities to platelet-derived growth factor (PDGF), a mitogen for connective tissue cells but not vascular endothelial cells from large vessels.

OBJECTS OF THE INVENTION

45 It is, accordingly, an object of the present invention to provide novel vascular endothelial growth factor C subunit DNA free of other mammalian DNA. Another object is to provide recombinant genes capable of expressing VEGF C subunit monomer or dimer. Another object is to provide vectors containing the DNA sequences for VEGF A or B plus C subunits. A further object is to provide a host cell transformed with a vector containing the DNA sequence for VEGF A or B plus C or VEGF C alone. It is also an object to provide a recombinant process for making VEGF C subunit. Another object is to provide a novel vascular endothelial cell growth factor which contains the C subunit. This may include heterodimers AC and BC and homodimer CC.

SUMMARY OF THE INVENTION

55 Vascular endothelial cell growth factor C subunit DNA is prepared by polymerase chain reaction techniques. The DNA encodes a protein that may exist as either a heterodimer or homodimer. The protein is a mammalian vascular endothelial cell mitogen and as such is useful for the promotion of vascular development and

repair. This unique growth factor is also useful in the promotion of tissue repair.

DETAILED DESCRIPTION

5 The present invention relates to a unique vascular endothelial cell growth factor (designated VEGF), isolated and purified from glioma cell conditioned medium, which exhibits mitogenic stimulation of vascular endothelial cells. Glioma is defined herein as any neoplasm derived from one of the various types of cells that form the interstitial tissue of the central nervous system including brain, spinal cord, posterior pituitary gland and retina. Consequently, the scope of the present invention is intended to include the unique growth factor
10 isolated and purified from any mammalian tissue or other cells including cell lines. Cell lines include, but are not limited to, glioma-derived cell lines such as C6, hs 683 and GS-9L; glioblastomas such as A-172 and T98G; neuroblastomas such as IMR-32 and SK-N-MC; neurogliomas such as H4; tetromas such as XB-2; astrocytomas such as U-87 MG and U-373 MG; embryonal carcinomas and non-transformed glial or astrocyte cell lines, and the human medulloblastoma line TE 671, with GS-9L and TE 671 being preferred. VEGF AB is present and can be isolated from rat tissue including ovary, heart and kidney. Anterior pituitary tumor cell lines such as GH3 and Hs 199 may also be used. It is intended that VEGF of this invention can be obtained from any mammal species capable of producing VEGF, this includes, but is not limited to, rat and human.

Vascular endothelial cell growth factor may exist in various microheterogeneous forms which are isolated from one or more of the various cells or tissues described above. Microheterogeneous forms as used herein
20 refer to a single gene product, that is a peptide produced from a single gene unit of DNA, which is structurally modified at the mRNA level or following translation. Peptide and protein are used interchangeably herein. The microheterogeneous forms will all have similar mitogenic activities. Biological activity and biologically active are used interchangeably and are herein defined as the ability of VEGF to stimulate DNA synthesis in target cells including vascular endothelial cells as described below which results in cell proliferation. The modifications
25 may take place either *in vivo*, or during the isolation and purification process. *In vivo* modification results from, but is not limited to, proteolysis, glycosylation, phosphorylation, deamidation or acetylation at the N-terminus. Proteolysis may include exoproteolysis wherein one or more terminal amino acids are sequentially, enzymatically cleaved to produce microheterogeneous forms which have fewer amino acids than the original gene product. Proteolysis may also include endoproteolytic modification that results from the action of endoproteases
30 which cleave the peptide at specific locations within the amino acid sequence. Similar modifications can occur during the purification process which also results in production of microheterogeneous forms. The most common modification occurring during purification is proteolysis which is generally held to a minimum by the use of protease inhibitors. Under most conditions one or more microheterogeneous forms are present following purification of native VEGFs. Native VEGFs refers to VEGF isolated and purified from cells that produce VEGFs.
35 Vascular endothelial cell growth factor may also exist in various alternatively spliced forms which is defined herein as the production of related mRNAs by differential processing of exons and introns. Exons are defined as those parts of the DNA sequence of a eukaryotic gene that code for the final protein product. It is also intended that the present invention includes VEGF subunits A,B and C which are defined as comprising the full length translation products of all alternatively spliced mRNAs made from the gene encoding the subunits and their corresponding mature amino acid sequences generated by proteolytic removal of the amino terminal secretory leader amino acid sequences. It is further intended that the invention only include those microheterogeneous and alternatively spliced VEGF subunits which when in the dimeric form exhibit biological activity such as vascular endothelial cell stimulation as discussed below.

Glioma cells such as the rat cell line GS-9L are grown to confluence in tissue culture flasks, about 175 cm²,
45 in a cell culture medium such as Dulbecco's Modified Eagle's Medium (DMEM) supplemented with about 10% newborn calf serum (NCS). When the cells reach confluence the culture medium is removed, the cell layers are washed with Ca⁺⁺, Mg⁺⁺-free phosphate buffered saline (PBS) and are removed from the flasks by treatment with a solution of trypsin, about 0.1%, and EDTA, about 0.04%. The cells, about 1 x 10⁸, are pelleted by centrifugation, resuspended in about 1500 ml of DMEM containing about 5% NCS and plated into a ten level cell
50 factory (NUNC), 6,000 cm² surface area. The cells are incubated for about 48 to about 96 hours, with 72 hours preferred, at about 37° C in an atmosphere of about 5% CO₂. Following incubation the medium is removed and the cell factories are washed about 3 times with PBS. About 1500 ml of fresh culture media is added containing about a 1:2 mixture of Ham's-F12/DMEM containing about 15 mM Hepes, pH about 7.4, about 5 µg/ml insulin, about 10 µg/ml transferrin and with or without about 1.0 mg/ml bovine serum albumin. This medium is replaced
55 with fresh medium after about 24 hr and collected every 48 hr thereafter. The collected conditioned medium is filtered through Whatmen #1 paper to remove cell debris and stored at about -20° C.

The GS-9L conditioned medium is thawed and brought to pH 6.0 with 1 M HCl. The initial purification step consists of cation exchange chromatography using a variety of cation exchangers on a variety of matrices such

as CM Sephadex C-50, Pharmacia Mono S, Zetachrom SP and Polyaspartic Acid WCX (Nest Group) with CM Sephadex C-50 (Pharmacia) being preferred. The VEGF-containing culture medium is mixed with CM Sephadex C-50 at about 2 gm per about 20 L of the conditioned medium and stirred at low speed for about 24 hr at 4° C. The resin is allowed to settle and the excess liquid is removed. The resin slurry is packed into a column and the remaining culture medium is removed. Unbound protein is washed from the column with 0.05 M sodium phosphate, about pH 6.0, containing 0.15 M NaCl. The VEGF AB is eluted with about 0.05 M sodium phosphate, about pH 6.0, containing about 0.6 M NaCl.

The active fractions collected from the CM Sephadex C-50 column are further fractionated by lectin affinity chromatography for additional purification of VEGF AB. The lectins which may bind VEGF AB include, but are not limited to, lectins which specifically bind mannose residues such as concanavalin A and lens culinaris agglutinin, lectins which bind N-acetylglucosamine such as wheat germ agglutinin, lectins that bind galactose or galactosamine and lectins which bind sialic acids, with concanavalin A (Con A) being preferred. A 0.9 cm diameter column containing about 5 ml packed volume of Con A agarose (Vector Laboratories) is washed and equilibrated with about 0.05 M sodium acetate, about pH 6.0, containing about 1 mM CaCl_2 , about 1 mM MnCl_2 and about 0.6 M NaCl. The unbound protein is washed from the column with equilibration buffer. The VEGF AB is eluted with about 0.1 M NaCl buffer containing about 0.32 M α -methyl mannoside and about 0.28 M α -methyl glucoside.

The VEGF AB active eluate from the Con-A column is applied to a Polyaspartic Acid WCX cation exchange high performance liquid chromatography (HPLC) column, 4.6 mm x 250 mm, pre-equilibrated in about 0.05 M sodium phosphate buffer, pH 6.0. The column is eluted with a linear gradient of about 0 to 0.75 M NaCl in the phosphate buffer over about 60 minutes. The flow rate is maintained at about 0.75 ml/min collecting 0.75 ml fractions. Vascular endothelial cell growth factor AB activity is present in fractions eluting between approximately 21.7 and 28.5 ml.

The active fractions eluted from the polyaspartic WCX column that contain VEGF AB are pooled, adjusted to about pH 7.0 and loaded onto a 1 x 10 cm column of Pharmacia Chelating Sepharose 6B charged with an excess of copper chloride and equilibrated in about 0.05 M sodium phosphate, about pH 7.0, containing about 2 M NaCl and about 0.5 mM imidazole (A buffer). VEGF AB is eluted from the column with a gradient from 0-20% B over 10 minutes, 20-35% B over 45 minutes and 35-100% B over 5 minutes at a flow rate of 0.3 ml/min, where B buffer is 0.05 M sodium phosphate, pH 7.0, containing about 2 M NaCl and 100 mM imidazole. The active fractions containing VEGF AB activity eluted between about 12.6 and 22.8 ml of the gradient effluent volume.

The pooled fractions containing VEGF AB activity eluted from the metal chelate column are loaded onto a 4.6 mm x 5 cm Vydac

C_4 reverse phase HPLC column (5 μm particle size) previously equilibrated in solvent A [0.1% trifluoroacetic acid (TFA)]. The column is eluted with a linear gradient of about 0 to 30% solvent B over 15 minutes, 30% B for an additional 15 minutes, then 30-45% B over 22.5 minutes and finally 45-100% B over 5.5 minutes. Solvent B consists of solvent A containing 67% acetonitrile (v/v). The flow rate is maintained at about 0.75 ml/min and fractions are collected every minute. The homogeneous VEGF AB elutes from the C_4 column under these conditions at between about 32 and about 38 ml of the gradient effluent volume.

Purity of the protein is determined by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE) in 12.5% crosslinked gels using the technique of Laemmli, Nature 227:680-684 (1970). The silver stained gels show VEGF AB to consist of one band under non-reducing conditions with an approximate apparent molecular mass of about 58,000 daltons. When a sample containing the microheterogeneous forms of VEGF AB is separated under reducing conditions it migrates as two about 23 kilodalton (kDa) subunits. The purification process results in VEGF AB that is essentially free of other mammalian cell products, such as proteins. Recombinantly derived VEGF AB will also be free of mammalian cell products.

Biological activity is determined by mitogenic assay using mammalian vascular endothelial cells. Human umbilical vein endothelial (HUVE) cells are plated on gelatin-coated dishes at a density of about 5000 cells per well in about 500 μl of Medium 199 (M199) containing about 20% heat-inactivated fetal calf serum (FCS). Samples to be assayed are added at the time of plating. The tissue culture plates are incubated at about 37° C for about 12 hours and about 2 microcuries of tritiated thymidine (NEN, 20 Ci/mmol) is added per ml of assay medium (1.0 μCi /well). The plates are incubated for a further 60 hr, the assay medium is removed and the plates are washed with Hanks balanced salt solution containing about 20 mM Hepes, about pH 7.5, and about 0.5 mg/ml bovine serum albumin. The cells are lysed and the labelled DNA solubilized with about 200 μl of a solution containing about 2 gm of sodium carbonate and about 400 mg sodium hydroxide in about 100 ml water. The incorporated radioactivity was determined by liquid scintillation counting. The concentration of VEGF which elicited a half-maximal mitogenic response in HUVE cells was approximately 2 ± 1 ng/ml. The glycosaminoglycan heparin, which is required in these assays at a level of 10-100 μg /ml to promote a response to a positive control, acidic fibroblast growth factor, does not enhance mitogenic stimulation of these cells by VEGF AB.

A purified about 1-2 µg sample of VEGF AB is reduced in about 0.1 M Tris, about pH 9.5, with about 0.1% EDTA, about 6 M guanidinium chloride and about 20 mM dithiothreitol for about 2 hr at about 50°C. The reduced protein is carboxymethylated for about 1 hour in a solution containing about 9.2 µM of unlabelled and 2.8 µM of ¹⁴C-iodoacetic acid in about 0.7 M Tris, about pH 7.8, and about 0.1% EDTA and about 6 M guanidinium chloride. The protein is carboxymethylated for about 1 hr at room temperature. The protein is isolated after reduction and carboxymethylation by reverse phase HPLC chromatography on a Vydac C₄ column, about 4.6 mm x 5 cm. The protein subunits are loaded onto a column pre-equilibrated with about 0.1% TFA and eluted by a 45 ml linear gradient from about 0.1% TFA to 0.1% TFA/67% acetonitrile at a flow rate of about 0.75 ml/min. The reduced and carboxymethylated protein eluted as two peaks at approximately 23 and 25 ml with the proportion being approximately equal as determined by monitoring absorbance at 210 nm.

Samples of the reduced and carboxymethylated monomers are applied to polybrene-coated glass fiber filters and their N-terminal sequences are determined by Edman degradation in an ABI gas phase microsequencer in conjunction with an ABI 120A on line phenylthiohydantoin analyzer following the manufacturers instructions. The protein showing the peak of absorbance eluting at approximately 25 ml (A subunit or monomer) yielded an amino terminal sequence of: SEQ ID NO:1

Ala Pro Thr Thr Glu Gly Glu Gln Lys Ala His Glu Val Val

which is identical to the A chain monomers of VEGF AA, Conn *et al.*, Proc. Natl. Acad. Sci. USA 87: 2628-2632 (1990). The peak of absorbance eluting at approximately 23 ml (B subunit or monomer) yielded an N-terminal sequence of: SEQ ID NO:2

Ala Leu Ser Ala Gly Asn Xaa Ser Thr Ser Thr Glu Met Glu Val Val

Pro Phe Asn Glu Val

plus a nearly equal amount of a truncated form of the same sequence missing the first three amino acid residues. The missing Xxx residue corresponds to an Asn residue in the cloned cDNA, see below. Since this missing Asn occurs in a classical Asn Xxx Ser/Thr N-glycosylation sequence it is presumed to be glycosylated. The A subunit and the total of both B subunits are recovered in nearly equal amounts supporting the interpretation that the two peptides combine to form an AB heterodimer in VEGF AB.

A sample of the A monomer was treated with either the protease trypsin which cleaves polypeptides on the C-terminal side of lysine and arginine residues or Lys C which cleaves polypeptides on the C-terminal side of lysine by procedures well known in the art. The peptides are isolated by reversed phase - HPLC(RP-HPLC). The amino acid sequences of the isolated peptides are determined using the Edman degradation in the ABI gas phase sequencer in conjunction with the ABI 120 A on line phenylthiohydantoin analyzer following manufacturer's instructions. The amino acid sequences are shown in Figure 1.

Reduced and carboxymethylated A monomer is dried and solubilized in about 0.7 M Tris, about pH 7.8, about 6 M guanidinium chloride containing about 0.1% EDTA. V8 protease is added in 0.1 M ammonium bicarbonate buffer, about pH 8.0, and the mixture is incubated for about 48 hr at about 37°C. The protease cleaves predominantly on the carboxyl terminal side of glutamic acid residues. The resulting polypeptides were resolved by C₁₈ RP-HPLC as above.

The reduced and carboxymethylated A subunit protein solution is adjusted to a pH of about 6.8 with 6 N HCl and dithiothreitol is added to a final concentration of 2 M for reduction of any methionine sulfoxide to methionine residues. After about 20 hr of reduction at about 39°C the protein is repurified by C₄ HPLC. The product is dried and cleaved on the carboxyl terminal side of methionine residues by 200 µl of 40 mM cyanogen bromide in about 70 % (v/v) formic acid under an argon atmosphere at about 20°C for about 24 hr in the dark. The cleavage products are resolved by C₁₈ RP-HPLC. The amino acid sequence is shown in Figure 1, see Conn *et al.*, Proc. Natl. Acad. Sci. USA 87:2628-2632 (1990).

The full length 190 amino acid residue protein translation product of the VEGF AB, A monomer or subunit, which is now known to be identical with the VEGF AA, A monomer, and its cDNA coding sequence are shown in Figures 2 and 6. The mature amino terminus begins at residue 27, immediately following a typical hydrophobic secretory leader sequence. A single potential N-glycosylation site exists at Asn₁₀₀. Most (143 amino acid residues) of the 164 residues of the reduced and carboxymethylated mature subunit including the amino terminus and HPLC reversed phase-purified products of tryptic (T), Lys-C (L), *Staphylococcus aureus* V8 protease (V8) and cyanogen bromide (CB) cleavages, were determined by direct microsequencing (Applied Biosystems 470A) using a total of 5 µg of protein. All residues identified by amino acid sequencing are denoted by arrows pointing to the right either directly beneath the mature processed sequence following the bracket at residue 27 for the amino terminal determination of the whole subunit or, for residues identified from the polypeptide cleavage products, above the double-headed arrows spanning the length of the particular polypeptide. One listed pair of polypeptides, V18A and V18B, was sequenced as a mixture and, therefore, are only confirmatory of the cDNA-deduced amino acid sequence, see Figures 1 and 5.

Samples of the reduced and carboxymethylated pure VEGF AB, A and B monomers, were each digested

with the Lys-C endoprotease, which cleaves polypeptides on the C-terminal side of lysine residues. The peptides were isolated by reverse phase HPLC and their amino acid sequences were determined as described above. The locations of the peptides in the final VEGF AB, A and B sequences are shown in Figure 2 and Figure 3, respectively.

5 The full length coding region of the A subunit or monomer is determined from three sets of overlapping cDNA clones. Degenerate oligonucleotide primers based on the amino acid sequences Phe-Met-Asp-Val-Tyr-Gln from polypeptide L42 (residues 42-47) and Cys-Lys-Asn-Thr-Asp from polypeptide T38 (residues 164-168) (see Figure 1) were used to PCR amplify the central region of the cDNA for VEGF A chain following the procedure of Saiki *et al.*, Science 230: 1350- 1354 (1985). A single band migrating at 420 bp was gel purified, digested with Sall, ligated into pGEM3Zf(+) and sequenced. The nucleotide sequence obtained (p4238) was used to design antisense and sense PCR primers to amplify the 5' and 3' ends of the cDNA according to the protocol described by Frohman *et al.* Proc. Natl. Acad. Sci. USA 85:8998-9002 (1988). These 5' and 3' clones are denoted p5-15 and pW3, respectively. Regions of complete DNA sequences, excluding the primers, determined for each set of clones are indicated by double-headed arrows above the nucleotide sequence. In addition to the cDNA coding the 164 amino acid secreted form identified by protein sequencing, two alternatively spliced cDNAs encoding a 146 amino acid and a 214 amino acid forms are cloned and sequenced, Figures 4, 5 and 6.

The full length coding region of the B subunit or monomer is determined from four sets of overlapping cDNA clones. Degenerate oligonucleotide primers based on the amino acid sequences from polypeptide L50 are used to PCR amplify the central region of the cDNA for VEGF AB, B monomer, following the procedure of Saiki *et al.*, Science 230: 1350-1354 (1985). A single band migrating at 108 bp was gel purified, digested with Sall, ligated into pGEM3Zf(+) and sequenced. The nucleotide sequence obtained (pYG) was used to design antisense and sense PCR primers to amplify the 5' and 3' ends of the cDNA according to the protocol described by Frohman *et al.* Proc. Natl. Acad. Sci. USA 85:8998-9002 (1988). These 5' and 3' clones are denoted p5V2 and p3V2, respectively. Additional 5' end sequences are determined from clone 202 isolated from a cDNA library prepared from GS-9L poly A⁺ RNA. Regions of complete DNA sequences, excluding the primers, determined for each set of clones are indicated by double-headed arrows above the nucleotide sequence. The entire base sequence for the 158 amino acid microheterogeneous B subunit and the 138 amino acid microheterogeneous B subunit are shown in Figures 7 and 8.

30 The full length coding region of the C subunit or monomer is determined from three sets of overlapping cDNA clones. Degenerate oligonucleotide primers based on the amino acid sequence Phe Ser Pro Ser Cys Val and Glu Met Thr Phe Ser Gly from rat VEGF B subunit are used to PCR amplify the central region of the cDNA of VEGF C chain following the procedure of Saiki *et al.*, Science 230: 1350-1354 (1985). A band migrating at 180 bp is gel purified, reamplified and digested with Sall, ligated into pGEM3Zf(+) and sequenced. The nucleotide sequence obtained (pFSEM') is used to design antisense and sense PCR primers to amplify the 5' and 3' ends of the cDNA according to the protocol described by Frohman *et al.*, Proc. Natl. Acad. Sci. USA 85: 8998-9002 (1988). The 5' and 3' clones are denoted p5:16 and p3:19, respectively. The entire base sequence and amino acid sequence for the C subunit are shown in Figure 9.

40 It is intended that vascular endothelial cell growth factor of the present invention exist as a heterodimer consisting of an A microheterogeneous and/or alternatively spliced subunit or a B microheterogeneous and/or alternatively spliced subunit combined with a C microheterogeneous and/or alternatively spliced subunit. It is further intended that VEGF homodimer of the present invention exist as two C subunits. The native forms of the A, B, C subunits may be processed form alternatively spliced full length translation products. The heterodimers or heterodimeric species can be depicted as: A+B, A+C or B+C with the A, B or C subunits existing in any of the alternatively spliced or microheterogeneous forms. The homodimers or homodimeric species can be formed by combinations of any of the alternatively spliced or microheterogeneous forms. It is also intended that the invention include all of the individual subunit forms of the A subunit, the B subunit and the C subunit of VEGF.

50 It is further intended that the nucleotide sequence for vascular endothelial cell growth factor be interpreted to include all codons that code for the appropriate amino acids in the sequence for each of the vascular endothelial growth factor subunits, as indicated by the degeneracy of the genetic code. It is further intended that the nucleotide sequence and the amino acid sequence for VEGF subunits include truncated genes or proteins that result in proteins which exhibits biological activity similar to vascular endothelial cell growth factor. The scope of the invention is intended to include all naturally occurring mutations and allelic variants and any randomly generated artificial mutants which may change the sequences but do not alter biological activity as determined by the ability to stimulate the division of vascular endothelial cells.

55 The above described heterodimers, homodimers and subunits of vascular endothelial cell growth factor are characterized by being the products of chemical synthetic procedures or of procaryotic or eucaryotic host

expression of the DNA sequences as described herein. A monomer is defined as a subunit that is not incorporated in an oligomeric unit. Expression of the recombinant VEGF genes (recombinant DNA) is accomplished by a number of different host cells which contain at least one of a number of expression vectors. Expression vectors are defined herein as DNA sequences that are required for the transcription of cloned copies of recombinant DNA sequences or genes and the translation of their mRNAs in an appropriate host. Such vectors can be used to express genes in a variety of hosts such as bacteria, bluegreen algae, yeast cells, insect cells, plant cells and animal cells, with mammalian cells being preferred. The genes may also be expressed using any of a number of virus expression systems. Specifically designated vectors allow the shuttling of DNA between bacteria-yeast, bacteria-plant or bacteria-animal cells. An appropriately constructed expression vector should contain: an origin of replication for autonomous replication in host cells, selective markers, a limited number of useful restriction enzyme sites, a high copy number, strong promoters and efficient translational stop signals. A promoter is defined as a DNA sequence that directs RNA polymerase to bind to DNA and to initiate RNA synthesis. A strong promoter is one which causes mRNAs to be initiated at high frequency. Expression vectors may include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses and cosmids. The expression of mammalian genes in cultured mammalian cells is well known in the art. Sambrook *et al.*, Molecular Cloning, A Laboratory Manual, 2nd Edition, Book 3, Cold Springs Harbor Laboratory Press (1989) and Current Protocols in Molecular Biology, Ausubel *et al.* Eds, Greene Publishing Associates and Wiley-Interscience, 1987 and supplements, disclose various mammalian expression vectors and vector systems along with methods for the introduction of recombinant vectors into mammalian cells. The cDNA for the monomeric forms of the A, B and C subunits can be expressed in a system such as that described by Linemeyer *et al.*, European Patent Application, Publication No. 259,953. The cDNA is incorporated into a commercially available plasmid such as pKK 223-3 (Pharmacia) as modified as by Linemeyer *et al.* and expressed in *E. coli*. Other expression systems and host cells are well known in the art.

The high Cys content and glycosylation sites of the A, B and C subunits along with the structure of the homo- and heterodimers suggest that expression of biologically active proteins can be carried out in animal cells. Expression may be carried out in Chinese hamster ovary (CHO) cells with the cloned VEGF DNA cotransfected with the gene encoding dihydrofolate reductase (dhfr) into dhfr- CHO cells, see Sambrook *et al.* Transformants expressing dhfr are selected on media lacking nucleosides and are exposed to increasing concentrations of methotrexate. The dhfr and VEGF genes are thus coamplified leading to a stable cell line capable of expressing high levels of VEGF. The plasmid is designed to encode either an A subunit, a B subunit or a C subunit or a combination of any two of these subunits. The two cDNAs are operably attached so that the protein produced will be dimeric and will have VEGF biological activity. Operably attached refers to an appropriate sequential arrangement of nucleotide segments, cDNA segments or genes such that the desired protein will be produced by cells containing an expression vector containing the operably attached genes, cDNA segments or nucleotides. Plasmids containing a single subunit species may be used to cotransfect a suitable cell line.

The expressed proteins (homodimers or heterodimers) are isolated and purified by standard protein purification processes. It is to be understood that the expression vectors capable of expressing heterodimeric forms of VEGF will contain two DNA sequences which will encode either an A subunit and/or a DNA sequence which will encode a B subunit and/or a DNA sequence which will encode a C subunit. Expression vectors capable of expressing homodimeric forms of VEGF will contain either one or two DNA sequences which encode either two A, two B or two C subunits.

The ability of the various species of VEGF to stimulate the division of vascular endothelial cells makes this protein in all microheterogeneous forms and alternative splicing forms useful as a pharmaceutical agent. The protein as used herein is intended to include all microheterogeneous forms as previously described. The protein can be used to treat wounds of mammals including humans by the administration of the novel protein to patients in need of such treatment.

The novel method for the stimulation of vascular endothelial cells comprises treating a sample of the desired vascular endothelial cells in a nutrient medium with mammalian VEGF, preferably human or rat, at a concentration of about 1-10 ng/ml. If the vascular endothelial cell growth is conducted *in vitro*, the process requires the presence of a nutrient medium such as DMEM or a modification thereof and a low concentration of calf or bovine serum such as about 0 to 2% by volume. Preservatives such as antibiotics may also be included; these are well known in the art.

The novel growth factors of this invention are useful for the coverage of artificial blood vessels with vascular endothelial cells. Vascular endothelial cells from the patient would be obtained by removal of a small segment of peripheral blood vessel or capillary-containing tissue and the desired cells would be grown in culture in the presence of VEGF and any other supplemental components that might be required for growth. After growth of adequate numbers of endothelial cells in culture to cover a synthetic polymeric blood vessel the cells would be plated on the inside surface of the vessel, such as fixed umbilical vein, which is then implanted in the patient.

Alternatively, tubular supports are coated in vitro with VEGF prior to implantation into a patient. Following implantation endothelial cells migrate into and grow on the artificial surface. Prior coating of the artificial vessel either covalently or noncovalently, with proteins such as fibrin, collagen, fibronectin or laminin would be performed to enhance attachment of the cells to the artificial surface. The cell-lined artificial vessel would then be surgically implanted into the patient and, being lined with the patient's own cells, would be immunologically compatible. The non-thrombogenic endothelial cell lining should decrease the incidence of clot formation on the surface of the artificial vessel and thereby decrease the tendency of vessel blockage or embolism elsewhere.

The novel proteins are also used for the production of artificial vessels. Vascular endothelial cells and smooth muscle cells from the patient would be obtained and grown separately in culture. The endothelial cells would be grown in the presence of VEGF as outlined above. The smooth muscle would be grown in culture by procedures well known in the art. A tubular mesh matrix of a biocompatible polymer (either a synthetic polymer, with or without a coating of proteins, or a non-immunogenic biopolymeric material such as surgical suture thread) would be used to support the culture growth of the smooth muscle cells on the exterior side and vascular endothelial cells on the interior surface. Once the endothelial cells form a confluent monolayer on the inside surface and multiple layers of smooth muscle cells cover the outside, the vessel is implanted into the patient.

The novel peptides can also be used for the induction of tissue repair or growth. The pure VEGF would be used to induce and promote growth of tissue by inducing vascular growth and/or repair. The peptide can be used either topically for tissue repair or intravascularly for vascular repair. For applications involving neovascularization and healing of surface wounds the formulation would be applied directly at a rate of about 10 ng to about 1 mg/cm²/day. For vascular repair VEGF is given intravenously at a rate of about 1 ng to about 100 µg/kg/day of body weight. For internal vascular growth, the formulation would be released directly into the region to be neovascularized either from implanted slow release polymeric material or from slow release pumps or repeated injections. The release rate in either case is about 10 ng to about 100 µg/day/cm³.

For non-topical application the VEGF is administered in combination with pharmaceutically acceptable carriers or diluents such as, phosphate buffer, saline, phosphate buffered saline, Ringer's solution, and the like, in a pharmaceutical composition, according to standard pharmaceutical practice. For topical application, various pharmaceutical formulations are useful for the administration of the active compound of this invention. Such formulations include, but are not limited to, the following: ointments such as hydrophilic petrolatum or polyethylene glycol ointment; pastes which may contain gums such as xanthan gum; solutions such as alcoholic or aqueous solutions; gels such as aluminum hydroxide or sodium alginate gels; albumins such as human or animal albumins; collagens such as human or animal collagens; celluloses such as alkyl celluloses, hydroxy alkyl celluloses and alkylhydroxyalkyl celluloses, for example methylcellulose, hydroxyethyl cellulose, carboxymethyl cellulose, hydroxypropyl methylcellulose, and hydroxypropyl cellulose; polyoxamers such as Pluronic® Polyols exemplified by Pluronic® F-127; tetronics such as tetronic 1508; and alginates such as sodium alginate.

The following examples illustrate the present invention without, however, limiting the same thereto.

EXAMPLE 1

Preparation of Medium Conditioned By GS-9L Cells

GS-9L cells were grown to confluence in 175 cm² tissue culture flasks in Dulbecco's Modified Eagle's Medium/10% newborn calf serum (DMEM/NCS). At confluence the medium was decanted from the flasks, the flasks were washed with calcium and magnesium free phosphate buffered saline (PBS) and the cells were removed by treatment with a 1X solution of trypsin/EDTA (Gibco). The cells (1×10^8) were pelleted by centrifugation, resuspended in 1500 ml of DMEM/5% NCS and plated into a ten level (6000 cm² surface area) cell factory (NUNC). After 72 hours incubation at 37° C in a 5% CO₂ atmosphere the medium was decanted and the cell factories were washed 3 times with PBS. The cells were refed with 1500 ml of a 1:2 mixture of Ham's F-12/DMEM containing 25 mM Hepes, pH 7.4, 5 µg/ml insulin, 10 µg/ml transferrin and 1.0 mg/ml bovine serum albumin. This medium was changed with fresh F-12/DMEM after 24 hours and collected every 48 hours after that. The conditioned medium was filtered through a Whatman #1 paper to remove cell debris and stored frozen at -20°C.

EXAMPLE 2

Carboxymethyl-Sephadex Chromatography of VEGF AA and VEGF AB

GS-9L conditioned medium, from Example 1, was thawed and brought to pH 6.0 with 1 M HCl. Two grams

of CM Sephadex C-50 cation exchange (Pharmacia) resin preequilibrated in PBS adjusted to pH 6.0 with 1 N HCl were added to 20 liters of conditioned medium. The mixture was stirred at low speed for 24 hours at 4° C. The resin was then allowed to settle and the medium was siphoned off. The remaining resin slurry was packed into a 3.0 cm diameter column and any remaining medium was allowed to drain off. Unbound protein was washed off the column with 0.05 M sodium phosphate, pH 6.0, containing 0.15 M NaCl. Vascular endothelial growth factor activity was eluted from the column with a subsequent wash of 0.05 M sodium phosphate, pH 6.0, containing 0.6 M NaCl.

EXAMPLE 3

Concanavalin A (Con A) Lectin Affinity Chromatography of VEGF AA and VEGF AB

A 0.9 cm diameter column containing about 5 ml of packed Con A agarose (Vector Laboratories) was equilibrated with 0.05 M sodium acetate, pH 6.0, containing 1 mM Ca^{++} , 1 mM Mn^{++} and 0.6 M NaCl. The active eluate from the CM Sephadex C-50 column, Example 2, was applied to the Con A agarose and unbound protein was washed from the column with equilibration buffer. The column was then rinsed with three column volumes of 0.05 M sodium acetate, pH 6.0, containing 1 mM Ca^{++} , 1 mM Mn^{++} and 0.1 M NaCl. Bound protein was subsequently eluted from the column by application of this buffer supplemented with 0.32 M α -methyl mannoside and 0.28 M α -methyl glucoside.

EXAMPLE 4

Polyaspartic Acid WCX HPLC Cation Exchange Chromatography of VEGF AA and VEGF AB

The active eluate from the Con A column, Example 3, was applied to a 25 cm x 4.6 mm poly(aspartic acid) WCX cation exchange HPLC column (Nest Group) pre-equilibrated in 0.05 M sodium phosphate buffer, pH 6.0. The column was eluted with a linear gradient of 0 to 0.75 M NaCl in this buffer over 60 minutes at a flow rate of 0.75 ml/min collecting 0.75 ml fractions. VEGF AB activity present in fractions eluting between approximately 21.7 and 28.5 ml were pooled.

EXAMPLE 5

Metal Chelate Chromatography

The active fractions eluted from the poly(aspartic acid) WCX column, Example 4, that contain VEGF AB were pooled, adjusted to pH 7.0 and loaded onto a 1 x 10 cm column of Pharmacia Chelating Sepharose 6B charged with an excess of copper chloride and equilibrated in 0.05 M sodium phosphate, pH 7.0, containing 2 M NaCl and 0.5 mM imidazole (A buffer). VEGF AB was eluted from the column with a gradient from 0-20% B over 10 minutes, 20-35% B over 45 minutes and 35-100% B over 5 minutes at a flow rate of 0.3 ml/min, where B buffer was 0.05 M sodium phosphate, pH 7.0, containing 2 M NaCl and 100 mM imidazole. The active fractions containing VEGF AB activity eluting between 12.6 and 22.8 ml of the gradient effluent volume were pooled.

EXAMPLE 6

Reverse Phase Chromatography

The fractions containing VEGF AB activity pooled from the metal chelate column, Example 5 were loaded onto a 4.6 mm x 5 cm Vydac C_4 reverse phase HPLC column (5 μm particle size) equilibrated in solvent A (0.1% trifluoroacetic acid (TFA)). The column was eluted with a gradient of 0-30% solvent B over 15 minutes, 30% B for an additional 15 minutes, then 30-45% B over 22.5 minutes and finally 45-100% B over 5.5 minutes where solvent B = A containing 67% acetonitrile. The flow rate was maintained at 0.75 ml/min. The active VEGF AB fractions eluting between approximately 32.2 and 37.5 ml of the gradient effluent volume were pooled.

EXAMPLE 7

Mitogenic Assays

Human umbilical vein endothelial cells (HUVE) were plated on gelatin-coated 48 well tissue culture dishes

at a density of 5000 cells/well in 500 μ l of Medium 199 containing 20% heat inactivated fetal calf serum (FCS). Samples to be assayed were added at the time of plating. The tissue culture plates are incubated at 37° C for 12 hours and 2 microcuries of tritiated thymidine (NEN, 20 Ci/mmol) was added per ml of assay medium (1.0 μ Ci/vell). The plates were incubated for a further 60 hr, the assay medium was removed and the plates were washed with Hanks balanced salt solution containing 20 mM Hepes, pH 7.5, and 0.5 mg/ml bovine serum albumin. The cells were lysed and the labelled DNA solubilized with 200 μ l of a solution containing 2 gm of sodium carbonate and 400 mg sodium hydroxide in 100 ml water. The incorporated radioactivity was determined by liquid scintillation counting.

The concentration of VEGF AB which elicited a half-maximal mitogenic response in HUVE cells was approximately 2 ± 1 ng/ml. The glycosaminoglycan heparin, which is required in these assays at a level of 10-100 μ g/ml to promote a response to a positive control, acidic fibroblast growth factor, does not enhance mitogenic stimulation of these cells by VEGF AB.

EXAMPLE 8

Purity And Protein Structural Characterization of VEGF AB

Purity of the protein under non-reducing conditions was determined by SDS-PAGE in 12.5% crosslinked gels according to the method of Laemmli, Nature 227: 680-685 (1970). The silver-stained gel contained a single band with an apparent mass of approximately 58 kDa. VEGF AB migrated in SDS-PAGE under reducing conditions in 15% crosslinked gels as a broad silver-stained band with apparent molecular mass of approximately 23 kDa.

VEGF AB was stored at 4°C in the aqueous trifluoroacetic acid (TFA)/acetonitrile mixture used to elute the homogeneous protein in reversed phase C_4 HPLC chromatography at the final stage of the purification protocol previously described. Aliquots of the purified protein (1-2 μ g) were vacuum evaporated to dryness in acid-washed 10 x 75 mm glass tubes and reduced for 2 hours at 50°C in 100 μ l of 0.1 M Tris buffer, pH 9.5, and 6 M guanidinium chloride containing 0.1% EDTA and 20 mM dithiothreitol (Calbiochem, Ultrol grade) under an argon atmosphere. The reduced protein was subsequently carboxymethylated for 1 hour at 20°C by the addition of 100 μ l of 0.7 M Tris, pH 7.8, containing 0.1 % EDTA, 6 M guanidinium chloride, 9.2 μ M unlabeled iodoacetic acid and 50 μ Ci of iodo[2- 14 C]acetic acid (17.9 mCi/mmol, Amersham). After completion of the carboxymethylation, the mixture was loaded directly onto a 4.6 mm x 5.0 cm Vydac C_4 column which had been preequilibrated in 0.1% TFA. The reduced and carboxymethylated protein was repurified by elution with a 45 minute linear gradient of 0 to 67% (v/v) acetonitrile in 0.1% TFA at a flow rate of 0.75 ml/min and stored in this elution solution at 4°C. The reduced and carboxymethylated protein eluted as two peaks at approximately 23 and 25 ml that were of approximately equal area as determined by monitoring absorbance at 210 nm.

Samples of the two protein subunits isolated after reduction and carboxymethylation were each applied to polybrene-coated glass fiber filters and their N-terminal sequences were determined by Edman degradation in an ABI gas phase microsequencer in conjunction with an ABI 120A on line phenylthiohydantoin analyzer following manufacturers instructions. The peak of absorbance eluting at approximately 25 ml (A subunit) yielded an amino terminal sequence Ala Pro Thr Thr Glu Gly Glu Gln Lys Ala His Glu Val Val SEQ ID NO: 1 identical to VEGF AA. The peak of absorbance eluting at approximately 23 ml (B subunit) yielded the N-terminal sequence Ala Leu Ser Ala Gly Asn Xaa Ser Thr Glu Met Glu Val Val Pro Phe Asn Glu Val SEQ ID NO: 2 plus a nearly equal amount of a truncated form of the same sequence missing the first three residues. The missing X residue corresponds to an Asn in the cloned sequence. Since this missing Asn occurs in a classical Asn-X-Ser/Thr N-glycosylation sequence it is presumed to be glycosylated. The A and sum of the B chain peptides were recovered in nearly equal amounts supporting the interpretation that the two peptides combine to form an AB heterodimer in VEGF II.

Reduced and carboxymethylated A and B subunits (650 ng each) were each dried by vacuum evaporation in acid-washed 10 x 75 mm glass tubes. Lys C protease (50 ng, Boehringer Mannheim), an enzyme that cleaves on the carboxyl terminal side of lysine residues, was added to each tube in 100 μ l of 25 mM Tris, pH 8.5, 0.1 % EDTA. The substrate protein subunits were separately digested at 37°C for 8 hours and the resulting polypeptides resolved by reversed phase HPLC chromatography on a 4.6 mm x 25 cm Vydac C_{18} column equilibrated in 0.1% TFA. Polypeptides were fractionated by elution with a 2 hour linear gradient of 0-67% acetonitrile in 0.1% TFA at a flow rate of 0.75 ml/min at 20°C. Individual peaks were manually collected and stored in this elution solution at 4°C.

The amino acid sequences of the isolated peptides were then determined using Edman degradation in an ABI gas phase sequencer in conjunction with the ABI 120 A on line phenylthiohydantoin analyzer (Applied Biosystems Int.). The peptide sequences are shown in the following Figures 2 and 3. The amino acid sequence

of Lys C fragment L20 (Fig. 5) demonstrates that the form of VEGF AB mature A subunit in the heterodimer is the 164 amino acid form. The amino acid sequence of Lys C fragment L26 (Fig. 3) demonstrates that the form of VEGF AB mature B subunit in the heterodimer is the 135 amino acid form derived from the 158 full length amino acid form.

5

EXAMPLE 9**Cloning and Sequencing of the VEGF A Monomer****10 PCR Amplification, Cloning and Sequencing of P4238**

Two degenerate oligonucleotides were synthesized in order to amplify the cDNA encoding the peptide sequences of VEGF A subunit between LysC fragment L 42 and tryptic fragment T38. These oligonucleotides were:

15

L42.2 5' TTTGTCGACTT[TC]ATGGA[TC]GT[N]TA[TC]CA 3'
SEQ ID NO:3

20

T383'B
5' CAGAGAATTCGTCGACA[AG]TC[N]GT[AG]TT[TC]TT
[AG]CA 3' SEQ ID NO:4

25

where N=ACGT

Poly A⁺ RNA was isolated from GS-9L cells using the Fast Track RNA isolation kit from Invitrogen and the protocol provided. First strand cDNA synthesis was performed as follows;

1 µg of GS-9L RNA was annealed to 1 µg of adapter primer TA17,

5' GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT 3' SEQ ID NO:5, by incubating in a volume of 10 µl at 70°C for 5 min. followed by cooling to room temperature. To this reaction was added:

30

3.0 µl water
2.5 µl 10X buffer (500 mM Tris-HCl pH 8.3, 750 mM KCl, 100 mM MgCl₂, 5 mM spermidine)
2.5 µl 100 mM DTT
2.5 µl 10 mM each dATP, dGTP, dCTP, dTTP
35 0.6 µl 15 units RNasin
2.5 µl 40 mM Na pyrophosphate
1.5 µl 15 units reverse transcriptase

and the reaction was incubated at 42°C for 1 hour, then diluted to 1 ml in 10 mM Tris-HCl 1 mM EDTA, pH 7.5.

40

PCR Reactions:**Primary reaction (100 µl)**

10 µl 10X buffer from Perkin Elmer Cetus GeneAmp kit
45 16 µl 1.25 mM each stock of dATP, dCTP, dGTP, and dTTP
2 µl first strand GS9L cDNA
2 µl 50 pMoles L42.2
2 µl 50 pMoles T383' B
0.5 µl 2.5 units Amplitaq DNA polymerase
50 67.5 µl water

Reaction conditions, 40 cycles of 94°C, 1'; 50°C, 2'30"; 72°C, 2'.

Prep scale secondary reaction:

55 100 µl 10X buffer
160 µl 1.25 mM each stock of dATP, dCTP, dGTP, and dTTP
10 µl primary PCR reaction
20 µl 500 pMoles L42.2

20 µl 500 pMoles T383'B
 5 µl 25 units Amplitaq DNA polymerase
 685 µl water

Reaction conditions 94°C, 1'; 55°C, 2'; 72°C, 2'; 30 cycles.

- 5 The PCR product was concentrated by Centricon 30 spin columns, purified on a 1% agarose gel, and digested with restriction endonuclease Sall. The Sall fragment was then ligated into Sall cut pGEM3Zf(+). The ligation mix was used to transform *E. coli* XL-1 blue. Plasmid DNA was isolated from white transformants and sequenced by the dideoxy chain termination method.

10 PCR Amplification, Cloning and Sequencing of pW-3

- Based on the sequence obtained from the p4238 clones, two specific PCR primers were synthesized; oligo 307 5' TTTGTGACTCAGAGCGGAGAAAGC 3' SEQ ID NO:6 and oligo 289 5' TTTGTGACGACAAAAT-CACTGTGAGC 3' SEQ ID NO:7. These primers were used in combination with oligoA 17
 15 5'GACTCGAGTCGACATCG 3' SEQ ID NO:8 to amplify the cDNA encoding the COOH terminus of VEGF A subunit using the 3' RACE technique described by Frohman *et al.*, PNAS 85: 8998-9002 (1988).

PCR reactions:

20 Primary reaction 100 µl

- 10 µl 10X buffer from Perkin Elmer Cetus GeneAmp kit
 18 µl 1.25 mM each stock of dATP, dCTP, dGTP, and dTTP
 0.35 µl first strand GS-9L cDNA
 2 µl 50 pMoles oligo 289
 25 0.5 µl 2.5 units Amplitaq DNA polymerase
 67.15µl water

Reaction conditions 94°C, 1'; 58°C, 2'; 72°C, 2'; 10 cycles then add 50 pMoles A17, then 1 cycle of 94°C, 1'; 58°C, 2'; 72°C, 40' followed by 40 cycles 94°C, 1'; 58°C, 2'; 72°C, 2'.

30 Prep Scale secondary reaction:

- 60 µl 10X buffer
 108 µl 1.25 mM each stock of dATP, dCTP, dGTP, and dTTP
 24 µl primary PCR reaction
 35 12 µl 300 pMoles oligo 307
 12 µl 300 pMoles oligo A17
 3 µl 15 units Amplitaq DNA polymerase
 381µl water

Reaction conditions 94°C, 1'; 58°C, 2'; 72°C, 2'; 30 cycles.

- 40 The PCR product was purified on a 1% agarose gel and digested with restriction endonuclease Sa1I. The Sa1I fragment was then ligated into Sa1I cut pGEM3Zf(+). The ligation mix was used to transform *E. coli* XL-1 blue. Plasmid DNA was isolated from white transformants and sequenced by the dideoxy chain termination method.

45 PCR Amplification, Cloning and Sequencing of p5-15

Based on the sequence of p4238 clones, two specific PCR primers were synthesized; oligo 113 5'TTTGTGACAACACAGGACGGCTTGAAG 3' SEQ ID NO:9 and oligo 74 5' TTTGTGACATACTCCTGGAAGATGTCC 3' SEQ ID NO*10.

- 50 These primers were used in combination with oligo A17 5' GACTCGAGTCGACATCG 3' SEQ ID NO:8 to amplify the cDNA encoding the amino terminus of VEGF A subunit using the 5' RACE technique described by Frohman *et al.*, supra. Oligo 151 was synthesized in order to specifically prime VEGF A subunit cDNA from GS-9L RNA. Oligo 151 is 5' CTTTCATCATTGCAGCAGC 3' SEQ ID NO:11.

- 55 RNA was isolated from GS-9L cells using the Fast Track RNA isolation kit from Invitrogen using the protocol provided. First strand cDNA synthesis was performed as follows;

One µg of GS9L RNA was annealed to 1 µg of oligo 151 by incubating in a volume of 6 µl at 70°C for 5' followed by cooling to room temperature. To this reaction was added:

- 1.5 μ l 10X buffer (500mM Tris-HCl, pH 8.3, 750 mM KCl, 100 mM MgCl₂, 5 mM spermidine)
 2.5 μ l 10 mM DTT
 2.5 μ l 10 mM each dATP, dGTP, dCTP, dTTP
 0.6 μ l 25 units RNasin
 5 2.5 μ l 40 mM Na pyrophosphate
 9.5 μ l 20 units diluted reverse transcriptase

The reaction was incubated at 42°C for 1 hour. Excess oligo151 was removed by Centricon 100 spin columns and the 5' end of the cDNA was tailed by the addition of dATP and terminal transferase. The tailed cDNA was diluted to a final volume of 150 μ l in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5.

10

PCR Reactions:

Primary reaction (50 μ l)

- 5 μ l 10X buffer from Perkin Elmer Cetus GeneAmp Kit
 15 8 μ l 1.25 mM each stock of dATP, dCTP, dGTP, and dTTP
 5 μ l first strand GS-9L cDNA prime with oligo 151 and tailed
 1 μ l 25 pMoles oligo 113
 1 μ l 25 pMoles oligo A17
 1 μ l 10 pMoles oligo TA17
 20 0.25 μ l 1.25 units AmpliTq DNA polymerase
 28.75 μ l water

Reaction conditions; 1 cycle 94°C 1'; 50°C 2'; 72°C 40' then 40 cycles of 94°C 1'; 50°C 1'30"; 72°C 2'

Prep scale secondary reaction:

25

- 60 μ l 10X buffer
 96 μ l 1.25 mM each stock of dATP, dCTP, dGTP, and dTTP
 6 μ l primary PCR reaction
 12 μ l 300 pMoles oligo74
 30 12 μ l 300 pMoles oligo A17
 3 μ l 15 units Amplitaq DNA polymerase
 411 μ l water

Reaction conditions 94°C, 1'; 55°C, 2'; 72°C, 2' 30 cycles.

- The PCR product was concentrated by Centricon 100 spin columns, and digested with restriction endonuclease Sall. The Sall fragment was then ligated into Sall cut pGEM3Zf(+). The ligation mix was used to transform E. coli XL-1 blue. Plasmid DNA was isolated from white transformants and sequenced by the dideoxy chain termination method. The base sequence is shown in Fig. 5.

35

Cloning and sequencing of alternative forms of VEGF A cDNA

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Based on the sequence obtained from the p5-15 and pW-3 clones, two specific PCR primers were synthesized; oligo 5' C 5' TTTGTCGACAACCATGAACCTTTCTGC 3' SEQ ID NO:12 and oligo 181 5' TTTGTCGACGGTGAGAGGTCTAGTTC 3' SEQ ID NO:13. These primers were used together to amplify multiple cDNAs encoding alternative forms of the VEGF A subunit.

45

Preparative PCR Reaction:

- 50 μ l 10X buffer
 80 μ l 1.25mM each stock of dATP, dCTP, dGTP, and dTTP
 50 10 μ l first strand GS-9L cDNA
 10 μ l 300pMoles oligo 5'C
 10 μ l 300pMoles oligo 181
 2.5 μ l 15 units Amplitaq DNA polymerase
 337.5 μ l water
 55 Reaction conditions 94°C, 1'; 58°C, 2'; 72°C, 3'; 40 cycles.

The PCR product was extracted with phenol/chloroform, concentrated by Centricon 30 spin columns, precipitated by ethanol, and digested with restriction endonuclease Sal I, and ligated into Sall cut pGEM3Zf(+). The ligation mix was used to transform E.coli XL-1 blue. Plasmid DNA was isolated from white transformants

and sequenced by the dideoxy chain termination method. Three sets of clones were identified. Clone#12 encoded the 190 amino acid form of VEGF A subunit identical to that shown in Fig. 1. The 164 amino acid secreted form of VEGF A subunit is that amino acid sequence running continuously from Ala²⁷ to Arg¹⁹⁰. Clone#14 has a 135 base pair deletion between the second base of the Asn¹⁴⁰ codon and the third base of the Arg¹⁸⁴ codon. This clone thus encodes a 146 aa form of the VEGF A subunit with the conversion of Asn¹⁴⁰ to Lys¹⁴⁰. The 120 amino acid secreted form of VEGF A subunit runs from Ala²⁷ to Asn¹⁴⁰, which becomes Lys¹⁴⁰ and does not begin until Cys¹⁸⁵, this form also finishes at Arg¹⁹⁰, Figure 4. Clone #16 has a 72 base pair insertion between the second and third base of the Asn¹⁴⁰ codon. This clone thus encodes the 214 amino acid form of the VEGF A subunit with the conversion of Asn¹⁴⁰ to Lys¹⁴⁰, Figure 6.

EXAMPLE 10

Cloning and Sequencing of the VEGF B Subunit

PCR Amplification, Cloning and Sequencing of pYG

Two degenerate oligonucleotides were synthesized in order to amplify the cDNA encoding the peptide sequences of VEGF B on Lys C fragment L50. These oligonucleotides were:

YI 5' TTTGTCGACATA[TC]AT[TC]A[GC][N]GA[TC]GA[AG]C 3' SEQ ID NO:14

GC 5' TTTGTCGACTC[AG]TC[AG]TT[AG]CA[AG]CA[N]CC 3' SEQ ID NO:15 where N=ACGT

RNA was isolated from GS-9L cells using the Fast Track RNA isolation kit from Invitrogen and the protocol provided. First strand cDNA synthesis was performed as follows;

1 µg of GS-9L poly A+RNA was annealed to 1 µg of adapter primer TA17,

5'GACTCGAGTCGACATCGATTTTTTTTTTTTTTTTTT 3' SEQ ID NO: 5, by incubating in a volume of 10 µl at 70°C for 5 min. followed by cooling to room temperature. To this reaction was added:

3.0 µl	water
2.5 µl	10X buffer (500 mM Tris-HCl, pH 8.3, 750 mM KCl, 100 mM MgCl ₂ , 5mM spermidine)
2.5 µl	100 mM DTT
2.5 µl	10 mM each dATP, dGTP, dCTP, dTTP
0.6 µl	15 units RNasin
2.5 µl	40 mM Na pyrophosphate
1.5 µl	15 units reverse transcriptase

and the reaction was incubated at 42°C for 1 hour, then diluted to 1 ml in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5.

PCR Reactions:

Primary reaction (50µl)

5 µl	10X buffer from Perkin Elmer Cetus GeneAmp kit
8 µl	1.25 mM each stock of dATP, dCTP, dGTP, and dTTP
1 µl	first strand GS-9L cDNA
1 µl	50 pMoles oligo YI
1 µl	50 pMoles oligo GC
0.25 µl	1.25 units Amplitaq DNA polymerase
33.75 µl	water

Reaction conditions, 40 cycles of 94°C, 1'; 50°C, 2'; 72°C, 2'.

Prep scale reaction:

60 µl	10X buffer
96 µl	1.25mM each stock of dATP, dCTP, dGTP, and dTTP
12 µl	first strand 659L cDNA
12 µl	500pMoles oligo YI
12 µl	500pMoles oligo GC
3 µl	15 units Amplitaq DNA polymerase
405 µl	water

Reaction conditions 94°C, 1'; 50°C, 2'; 72°C, 2' 40 cycles.

The PCR product was concentrated by Centricon 30 spin columns and digested with restriction endonuc-

lease Sall. The Sall fragment was then ligated into Sall cut pGEM3Zf(+). The ligation mix was used to transform *E. coli* XL-1 blue. Plasmid DNA was isolated from white transformants and sequenced by the dideoxy chain termination method.

5 PCR Amplification, Cloning and Sequencing of p3V2

Based on the sequence obtained from the pYG clones, a specific PCR primer was synthesized; oligo HP 5' TTTGTGCGACACACCCTAATGAAGTGTC 3' SEQ ID NO:16.

This primer was used in combination with oligo A17 5'

10 GACTCGAGTCGACATCG 3' SEQ ID NO:8 to amplify the cDNA encoding the COOH terminus of the VEGF B subunit using the 3' RACE technique described by Frohman *et al.*, PNAS 85: 8998-9002 (1988).

Preparative PCR reaction:

15 60 µl 10X buffer from Perkin Elmer Cetus Gene Amp Kit
12 µl first strand 659L cDNA
96 µl 1.25 mM each of dATP, dCTP, dGTP, dTTP
12 µl 300 pMoles oligo A17
12 µl 300 pMoles oligo HP
20 3 µl 15units Amplitaq DNA polymerase
405 µl water

Reaction conditions 1 cycle of 94°C, 1'; 58°C, 2'; 72°C, 2'; followed by 40 cycles 94°C, 1', 58°C, 2' and 72°C, 2'.

25 The PCR product was concentrated by Centricon 30 spin columns, precipitated with ethanol and digested with restriction endonuclease Sall. The Sall fragment was then ligated into Sall cut pGEM3Zf(+). The ligation mix was used to transform *E. coli* XL-1 blue. Plasmid DNA was isolated from white transformants and sequenced by the dideoxy chain termination method.

PCR Amplification, Cloning and Sequencing of p5V2

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Based on the sequence of pYG clones, two specific PCR primers were synthesized; oligoVL'

5' TTTGTGCGACAACAGCGACTCAGAAGG 3' SEQ ID NO: 17 and oligoVS' 5' TTTGTGCGACACTGAATATATGAGACAC 3' SEQ ID NO:18. These primers were used in combination with oligo A17

35 5' GACTCGAGTCGACATCG 3' SEQ ID NO:8 to amplify the cDNA encoding the amino terminus of the VEGF B subunit using the 5' RACE technique described by Frohman *et al.*, supra. Oligo 151 was synthesized in order to prime cDNA from GS-9L RNA Oligo 151 is 5' CTTTCATCATTGCAGCAGC 3' SEQ ID NO:11.

Poly A+RNA was isolated from GS9L cells using the Fast Track RNA isolation kit from Invitrogen using the protocol provided. First strand cDNA synthesis was performed as follows:

40 One µg of GS9L RNA was annealed to 1 µg of oligo 151 by incubating in a volume of 6 µl at 70°C for 5' followed by cooling to room temperature. To this reaction was added:
1.5 µl 10X buffer (500 mM Tris-HCl, pH 8.3, 750 mM KCl, 100 mM MgCl₂, 5mM spermidine)
2.5 µl 10 mM DTT
2.5 µl 10 mM each dATP, dGTP, dCTP, dTTP
45 0.6 µl 25 units RNasin
2.5 µl 40 mM Na pyrophosphate
9.5 µl 20 units diluted reverse transcriptase

The reaction was incubated at 42°C for 1 hour.

50 Excess oligo 151 was removed by Centricon 100 spin columns and the 5' end of the cDNA was tailed by the addition of dATP and terminal transferase. The tailed cDNA was diluted to a final volume of 150 µl in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5

PCR Reactions:

55 Primary reaction (50 µl)

5 µl 10X buffer from Perkin Elmer Cetus GeneAmp Kit
8 µl 1.25 mM each stock of dATP, dCTP, dGTP, and dTTP
5 µl first strand GS9L cDNA primed with oligo151 and tailed

- 1 μ l 25 pMoles oligo VL'
 1 μ l 25 pMoles oligo A17
 1 μ l 10 pMoles oligo TA17
 0.25 μ l 1.25 units AmpliQ DNA polymerase
 5 28.75 μ l water
 Reaction conditions; 1 cycle 94°C, 1'; 58°C, 2'; 72°C, 40' then 40 cycles of 94°C, 1'; 58°C, 2'; 72°C, 2'.

Prep scale secondary reaction

- 10 100 μ l 10X buffer
 160 μ l 1.25 mM each stock of dATP, dCTP, dGTP, and dTTP
 10 μ l primary PCR reaction
 20 μ l 500 pMoles oligo VS'
 20 μ l 300 pMoles oligo A17
 15 5 μ l 25 units AmpliQ DNA polymerase
 685 μ l water

Reaction conditions 94°C, 1'; 58°C, 2'; 72°C, 2' 30 cycles.

- The PCR product was extracted with phenol/chloroform, concentrated by Centricon 30 spin columns, precipitated by ethanol, and digested with restriction endonuclease Sall. The Sall fragment was purified on 4%
 20 Nu-Sieve Agarose gel then ligated into Sall cut pGEM3Zf(+). The ligation mix was used to transform *E. coli* XL-1 blue. Plasmid DNA was isolated from white transformants and sequenced by the dideoxy chain termination method.

PCR Amplification, Cloning and Sequencing of pCV2 and pCV2.1

- 25 Based on the sequences of the p3V2 and p5CV2 clones, two specific PCR primers were synthesized; oligo 5'CV2.1
 5' TTTGTCGAC[N][N]GCAGGTCCTAGCTG 3' SEQ ID NO:19 and oligo 3'CV2 5'
 TTTGTCGAC[N][N]CTAATAAATAGAGGG 3' SEQ ID NO:20.
 30 These primers were used together to amplify the cDNA encoding the VEGF B subunit.

Preparative PCR Reaction:

- 40 μ l 10X buffer
 35 64 μ l 1.25 mM each dATP, dTTP, dGTP, dCTP
 8 μ l first strand GS-9L cDNA
 8 μ l 200 pMoles 5'CV2.1
 8 μ l 200 pMoles 3'CV2
 2 μ l 10units AmpliQ DNA polymerase
 40 270 μ l water
 Reaction conditions: 94°C, 1', 58°C, 2', 72°C, 2'; 40 cycles.

- The PCR product was extracted with phenol/chloroform, concentrated by Centricon 30 spin columns, precipitated by ethanol, and digested with restriction endonuclease Sal I, and ligated into Sal I cut pGEM3Zf(+). The ligation mix was used to transform *E. coli* XL-1 blue. Plasmid DNA was isolated from white transformants
 45 and sequenced by the dideoxy chain termination method. Two sets of clones were identified, one encoded a 158 amino acid sequence and the other encoded a 138 amino acid sequence, see Figures 7 and 8.

cDNA Cloning of VEGF B Subunit

- 50 The DNA and protein sequences for the amino terminus of the signal peptide of VEGF B was determined from a cDNA clone isolated from a cDNA library constructed from GS-9L polyA+ RNA.

First Strand Synthesis

- Anneal 15.6 μ l (5ug) GS-9L polyA+ RNA and 2.5 μ l (2.5ug) oligo dT-XbaI primer by heating to 70° C 5' slow cool to room temperature. Add the following:
 55 5.5 μ l 10X buffer (500 mM Tris-HCl, pH 8.3 (42° C), 750 mM KCl, 100 mM MgCl₂, 5mM spermidine
 5.5 μ l 100mM DTT
 5.5 μ l 10 mM each dATP, dTTP, dCTP, dGTP
 1.4 μ l (55units) RNasin

5.5 µl 40mM NaPPi
 13.5 µl (55units) reverse transcriptase
 Incubate at 42° C 60'.

5 Second Strand Synthesis:

Assemble reaction mix

50 µl first strand reaction
 25 µl 10X buffer (500 mM Tris-HCl, pH7.2, 850 mM KCL, 30 mM MgCl₂ 1mg/ml BSA, 100 mM (NH₄)₂SO₄)
 10 7.5 µl 100 mM DTT
 25 µl 1mM NAD
 6.5 µl (65units) *E. coli* DNA Polymerase I
 2.5 µl (2.5units) *E. coli* DNA Ligase
 2.5 µl (2 units) *E. coli* RNase H
 15 135 µl water

Incubate at 14° C for 2h and then incubate 70° C for 10'. Add 1ul (10 units) T4 DNA Polymerase, incubate at 37° C for 10', add 25 µl 0.2M EDTA an extract with phenol/chloroform, then precipitate by the addition of 0.5 volume of 7.5 M ammonium acetate and 3 volumes of ethanol, collect precipitate and resuspend in 20 µl of 10 mM Tris-HCl, pH 7.5, 1mM EDTA.

20 cDNA Library Construction

The above cDNA was ligated into EcoRI/ XbaI digested LambdaGEM-4 (Promega Biochemicals) after the addition of EcoRI linkers and digestion with EcoRI and XbaI. A cDNA library was amplified from ~50, 000 independent clones.

Isolation of Rat VEGF B cDNA Clone

25 The above cDNA library was screened by plaque hybridization using pCV2 as a probe. Hybridization conditions were as follows:

5XSSC (1XSSC is 0.15M sodium chloride, 0.015M sodium citrate,

50% Formamide

5X Denhardt's Solution (1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin)

30 0.15 mg/ml salmon sperm DNA hybridize overnight at 42° C.

Filters were washed 3 times in 2XSSC, 0.1% SDS at room temperature for 5', then 1 time in 1XSSC, 0.1% SDS at 50C for 30'. Positive clones were identified by autoradiography.

The DNA from phage #202 was digested with restriction endonuclease SpeI and the 1.1kb band ligated into XbaI digested pGEM3Zf(+). The ligation mix was used to transform *E. coli* XL-1 blue. Plasmid DNA was isolated from white transformants and sequenced by the dideoxy chain termination method. The cDNA sequence and predicted amino acid sequence of the signal peptide are shown in Figures 7 and 8.

The entire nucleotide and amino acid sequence of the 138 amino acid form is shown in Fig. 7. The secreted protein starts at Ala²⁴ and continues to Arg¹³⁸. The entire nucleotide and amino acid sequence of the 158 amino acid form is shown in Figures 8. The secreted protein starts at Ala²⁴ and continues to Leu¹⁵⁸.

40 EXAMPLE 11

Cloning and sequencing VEGF C Subunit

45 PCR Amplification, Cloning and Sequencing of pFSEM'

Two degenerate oligonucleotides were synthesized based on the sequence of rat VEGF B monomer in order to amplify VEGF cDNAs from the human medulloblastoma line TE-671, ATCC HTB (McAllister *et al.*, Int. J. Cancer 20:206-212 [1977]). These oligonucleotides were:

50 FS 5'TTTGTCGACA TTC AGT CC(N) TC(N) TG(TC) GT 3' SEQ ID NO:21

EM' 5' TTTGTCGACA CTG AGA GAA (N)GT CAT (CT)TC 3'

SEQ ID NO:22

where N= AGCT

55 Poly A+ RNA was isolated from TE-671 cells using the Fast Track RNA isolation kit from Invitrogen and the protocol provided. First strand cDNA synthesis was performed as follows using the cDNA Cycle kit from Invitrogen;

1 µl 1 µg of TE-671 polyA+ RNA

19 µl water

- 5 μ l 100mM MeMgOH
 6.25 μ l 0.7M B-mercaptoethanol
 2.5 μ l random primer
 2.5 μ l RNase Inhibitor
 5 10 μ l 5X RT buffer
 2.5 μ l 25mM dNTPs
 1.25 μ l reverse transcriptase 12.5units

The reaction was incubated for 60' at 42°C, then 3' at 95°C, placed on ice, then an additional 1.25 μ l reverse transcriptase was added and the reaction incubated an additional 60' at 42°C.

- 10 The above procedure was performed in duplicate and the cDNAs pooled to a final volume of 100 μ l.

PCR Reactions:

Primary reaction (100 μ l)

- 15 10 μ l 10X buffer from Perkin Elmer Cetus GeneAmp kit
 16 μ l 1.25mM each of dATP, dCTP, dGTP, TTP
 10 μ l first strand TE-671 cDNA
 2 μ l 50 pmoles FS primer
 2 μ l 50 pmoles EM' primer
 20 0.5 μ l 2.5 units Amplitaq DNA polymerase
 59.5 μ l water

Reaction conditions: 40 cycles of 90°C, 1'; 2' ramp to 45°C; 2' at 45°C; 2' at 72°C.

Gel Purification

- 25 20 μ l of the primary PCR reaction was purified on a 4% NuSieve agarose gel. The 180 base pair band was excised from the gel, heated to 65°C for 5' and used directly as template for the secondary PCR reaction.

Secondary PCR reaction 200 μ l

- 20 μ l 10X buffer from Perkin Elmer Cetus GeneAmp kit
 32 μ l 1.25mM each of dATP, dCTP, dGTP, TTP
 5 μ l melted gel slice
 30 4 μ l 100 pmoles FS primer
 4 μ l 100 pmoles EM' primer
 1 μ l 5 units Amplitaq DNA polymerase
 134 μ l water

Reaction conditions: 35 cycles of 94°C, 1'; 50°C, 2'; 72°C, 2'

- 35 The PCR product was purified on a Qiagen tip 5 column, then digested with restriction endonuclease Sall. The Sall fragment was then ligated into Sall cut pGEM3Zf(+), and the ligation mix used to transform E. coli XL-1 blue. Plasmid DNA was isolated from white transformants and sequenced by the dideoxy chain termination method.

40 PCR Amplification, Cloning and Sequencing of p3'.19

Based on the sequence obtained from the pFSEM' clone, a specific PCR primer was synthesized; oligo LH 5' TTTGTCGACA CTG CAC TGT GTG CCG GTG 3' SEQ ID NO:23. This primer was used in combination with oligo A17, 5' GACTCGAGTCGACATCG 3' SEQ ID NO:24, to amplify the cDNA encoding the COOH terminus of the VEGF C subunit using the 3' RACE technique described by Frohman et al., PNAS 85:8998-9002 (1988).

Poly A+ RNA was isolated from TE-671 cells using the Fast Track RNA isolation kit from Invitrogen and the protocol provided. First strand cDNA synthesis was performed as follows using the cDNA Cycle kit from Invitrogen and the TA17 adapter primer.

- 50 TA17 5' GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT 3' SEQ ID NO:5
 0.8 μ l 1 μ g of TE-671 polyA+ RNA
 20.7 μ l water
 5 μ l 100 mM MeMgOH
 6.25 μ l 0.7 M B-mercaptoethanol
 55 1.0 μ l 0.88 μ g primer TA17
 2.5 μ l RNase Inhibitor
 10 μ l 5X RT buffer
 2.5 μ l 25mM dNTPs

1.25 μ l reverse transcriptase 12.5 units

The reaction was incubated for 60' at 42°C, then 3' at 95°C, placed on ice, then an additional 1.25ul reverse transcriptase was added and the reaction incubated an additional 60' at 42°C.

5 3' RACE PCR

20 μ l 10 X buffer from Perkin Elmer Cetus GeneAmp kit
32 μ l 1.25mM each of dATP, dCTP, dGTP, TTP
20 μ l first strand TE-671 cDNA primed with TA17
10 2 μ l 50 pmoles LH primer
2 μ l 50 pmoles A17 primer
1.0 μ l 5 units Amplitaq DNA polymerase
123 μ l water

Reaction conditions: 40 cycles of 94C, 1'; 2' at 58°C; 3' at 72°C.

15 The PCR product was purified on a Qiagen tip 5 column, then digested with restriction endonuclease Sall. The Sall fragment was then ligated into Sall cut pGEM3Zf(+), and the ligation mix used to transform *E. coli* XL-1 blue. Plasmid DNA was isolated from white transformants and sequenced by the dideoxy chain termination method.

20 PCR Amplification, Cloning and Sequencing of p5'.16

Based on the sequence obtained from the pFSEM' clone, two specific PCR primers were synthesized; oligo VE' 5' TTTGTCGACA AC ATT GGC CGT CTC CAC C 3' SEQ ID NO:24, and oligo TG' 5' TTTGTCGACA ATC GCC GCA GCA GCC GGT 3' SEQ ID NO:25. These primers were used in combination with

25 oligo A17, 5' GACTCGAGTCGACATCG 3' SEQ ID NO:8, and oligo TA17 5'GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT 3' SEQ ID NO:5 to amplify the cDNA encoding the amino terminus of the VEGF C subunit using the 5' RACE technique described by Frohman et al., PNAS 85: 8998-9002 (1988).

30 Poly A+ RNA was isolated from TE-671 cells using the Fast Track RNA isolation kit from Invitrogen and the protocol provided. First strand cDNA synthesis was performed as follows using the cDNA Cycle kit from Invitrogen and the VE' primer:

1.0 μ l 1 μ g of TE-671 polyA+ RNA
20.25 μ l water
5 μ l 100 mM MeMgOH
35 6.25 μ l 0.7 M B-mercaptoethanol
1.0 μ l 1.0 μ g primer VE'
2.5 μ l RNase Inhibitor
10 μ l 5X RT buffer
2.5 μ l 25 mM dNTPs
40 0.5 μ l AMV reverse transcriptase (Promega) 10units

The reaction was incubated for 60' at 42°C, then 3' at 95°C, placed on ice, then an additional 1.25ul reverse transcriptase was added and the reaction incubated an additional 60' at 42°C. Excess oligo VE' was removed by a Centricon 100 spin column and the 5' end of the cDNA was tailed by the addition of dATP and terminal transferase. The tailed cDNA was diluted to a final volume of 200 ul in 10mM Tris-HCl, 1mM EDTA, pH 7.5.

45

5' RACE PCR 5 X 100ul

10 μ l 10X buffer from Perkin Elmer Cetus GeneAmp kit
16 μ l 1.25mM each of dATP, dCTP, dGTP, TTP
50 10 μ l first strand TE-671 cDNA primed with VE'
2 μ l 50 pmoles TG' primer
2 μ l 50 pmoles A17 primer
2 μ l 20 pmoles TA17 primer
0.5 μ l 2.5 units AMplitaq DNA polymerase
55 57.5 μ l water

Reaction conditions: 40 cycles of 94°C, 1'; 2' ramp to 58°C; 2' at 58°C; 2' at 72°C.

The PCR product was purified on a Qiagen tip 5 column, then digested with restriction endonuclease Sall. The Sall fragment was then ligated into Sall cut pGEM3Zf(+), and the ligation mix used to transform *E. coli*

XL-1 blue. Plasmid DNA was isolated from white transformants and sequenced by the dideoxy chain termination method. The combined sequences from plasmids pFSEM', p3'19 and p5'16 are shown in Figure 9.

PCR Amplification, Cloning and Sequencing of phVC16 and phVC2

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Based on the sequences of the p5'. 16 and p3'. 19 clones, two specific PCR primers were synthesized; oligo 5' GCVB 5' TTTGTCGAC TGG CTC TGG ACG TCT GAG 3' SEQ ID NO:26 and oligo 3'VC 5' TTTGTCGAC ACT GAA GAG TGT GAC GG 3' SEQ ID NO:27. These primers were used together to amplify the cDNA encoding the complete VEGF C subunit.

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Poly A+ RNA was isolated from TE-671 cells using the Fast Track RNA isolation kit from Invitrogen and the protocol provided. First strand cDNA synthesis was performed as follows using the cDNA Cycle kit from Invitrogen;

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0.8µl 1µg of TE-671 polyA+ RNA

19.2µl water

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5µl 100 mM MeMgOH

6.25µl 0.7 M B-mercaptoethanol

2.5µl oligo dT primer

2.5µl RNase Inhibitor

10µl 5X RT buffer

25

2.5µl 25 mM dNTPs

1.25µl reverse transcriptase 12.5units

The reaction was incubated for 60' at 42C, then 3' at 95C, placed on ice, then an additional 1.25ul reverse transcriptase was added and the reaction incubated an additional 60' at 42C.

PCR Reaction 200 ul

25

20µl 10X buffer from Perkin Elmer Cetus GeneAmp kit

32µl 1.25mM each of dATP, dCTP, dGTP, TTP

20µl first strand TE-671 cDNA primed with oligo dT

4µl 50 pmoles 5' GCVB primer

4µl 50 pmoles 3'VC primer

30

1µl 5 units Amplitaq DNA polymerase

119µl water

Reaction conditions: 40 cycles of 94°C, 1'; 2' at 50°C; 2' at 72°C.

The PCR product was purified on a Qiagen tip 5 column, then digested with restriction endonuclease Sall.

The Sall fragment was then ligated into Sall cut pGEM3Zf(+), and the ligation mix used to transform *E. coli*

35

XL-1 blue. Plasmid DNA was isolated from white transformants and sequenced by the dideoxy chain termination method. In the sequences of clones phVC16 and phVC2 base 463 (Fig. 9) was changed from a T to a C eliminating the translational stop codon following amino acid 154; this results in the addition of 16 amino acids following amino acid Lys 154. The nucleotide sequence and the deduced amino acid sequence of this addition is:

40

CAG AGA CCC ACA GAC TGC CAC CTG TGC GGC GAT GCT GTT

Gln Arg Pro Thr Asp Cys His Leu Cys Gly Asp Ala Val

155

160

165

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CCC OGG AGG TAA

Pro Arg Arg

170

SEQ ID NO:29

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In addition clone phVC16 contains a 3 base pair deletion (Figure 9, nucleotide residues 73-75) resulting in the deletion of Gln 25.

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SEQUENCE LISTING

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

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(A) LENGTH: 14 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: Not Applicable

(D) TOPOLOGY: linear

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(x1) SEQUENCE DESCRIPTION: SEQ ID NO:1
Ala Pro Thr Thr Glu Gly Glu Gln Lys Ala His Glu Val
      5              10
Val

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: Not Applicable

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:
Ala Leu Ser Ala Gly Asn Xaa Ser Thr Glu Met Glu Val
 5 10
Val Pro Phe Asn Glu Val
 15

(2) INFORMATION FOR SEO ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ
TTTGTCTGACT TYATGGAYGT NTAYCA 26

(2) INFORMATION FOR SEQ ID NO:4:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CAGAGAATTC GTCGACARTC NGTRTTYTTR CA 32

(2) INFORMATION FOR SEO ID NO:5:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GACTCGAGTC GACATCGATT TTTTTTTTTT TTTT 35

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

- (D) TOPOLOGY: linear
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
 TTTGTCGACA ACACAGGACG GCTTGAAG 28
- 5
- (2) INFORMATION FOR SEQ ID NO:7:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 25 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:
 TTTGTCGACG AAAATCACTG TGAGC 25
- 10
- (2) INFORMATION FOR SEQ ID NO:8:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 17 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:
 GACTCGAGTC GACATCG 17
- 15
- (2) INFORMATION FOR SEQ ID NO:9:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 28 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:
 TTTGTCGACA ACACAGGACG GCTTGAAG 28
- 20
- (2) INFORMATION FOR SEQ ID NO:10:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 28 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:
 TTTGTCGACA TACTCCTGGA AGATGTCC 28
- 25
- (2) INFORMATION FOR SEQ ID NO:11:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 18 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:
 CTTTCATCATT GCAGCAGC 18
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- (2) INFORMATION FOR SEQ ID NO:12:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:
TTTGTGACA ACCATGAAC TTCTGC 26
 - (2) INFORMATION FOR SEQ ID NO:13:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:
TTTGTGACG GTGAGAGGTC TAGTTC 26
 - (2) INFORMATION FOR SEQ ID NO:14:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:
TTTGTGACA TAYATHGCNG AYGARC 26
 - (2) INFORMATION FOR SEQ ID NO:15:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:
TTTGTGACT CRTCRITRCA RCANCC 26
 - (2) INFORMATION FOR SEQ ID NO:16:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:
TTTGTGACA CACCCTAATG AAGTGTC 27
 - (2) INFORMATION FOR SEQ ID NO:17:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single

(D) TOPOLOGY: linear
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:
 TTTGTCGACA ACAGCGACTC AGAAGG 26

5 (2) INFORMATION FOR SEQ ID NO:18:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 27 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 10 (D) TOPOLOGY: linear
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:
 TTTGTCGACA CTGAATATAT GAGACAC 27

(2) INFORMATION FOR SEQ ID NO:19:
 15 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 25 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:
 TTTGTCGACN NGCAGGTCCT AGCTG 25

(2) INFORMATION FOR SEQ ID NO:20:
 25 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 26 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:
 TTTGTCGACN NCTAATAAAT AGAGGG 26

(2) INFORMATION FOR SEQ ID NO:21:
 (i) SEQUENCE CHARACTERISTICS:
 35 (A) LENGTH: 27 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:
 40 TTTGTCGACA TTCAGTCCNT CNTGYGT 27

(2) INFORMATION FOR SEQ ID NO:22:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 28 base pairs
 45 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:
 TTTGTCGACA CTGAGAGAAN GTCATYTC 28

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- (2) INFORMATION FOR SEQ ID NO:23:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:
TTTGTGCGACA CTGCACTGTG TGCCGGTG 28
 - (2) INFORMATION FOR SEQ ID NO:24:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:
TTTGTGCGACA ACATTGGCCG TCTCCACC 28
 - (2) INFORMATION FOR SEQ ID NO:25:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:
TTTGTGCGACA ATCGCCGCAG CAGCCGGT 28
 - (2) INFORMATION FOR SEQ ID NO:26:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:
TTTGTGCGACT GGCTCTGGAC GTCTGAG
 - (2) INFORMATION FOR SEQ ID NO:27:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:
TTTGTGCGACA CTGAAGAGTG TGACGG 26
 - (2) INFORMATION FOR SEQ ID NO:28:
 - (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 51 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

5 CAG AGA CCC ACA GAC TGC CAC CTG TGC GGC GAT
GCT GTT 39

CCC CGG AGG TAA
51

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(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16

(B) TYPE: amino acids

15

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Gln Arg Pro Thr Asp Cys His Leu Cys Gly Asp
20 Ala Val

5

10

Pro Arg Arg
15

25

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 577 base pairs

(B) TYPE: nucleic acid

30

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

AACC

4

35 ATG AAC TTT CTG CTC TCT TGG GTG CAC TGG ACC CTG GCT TTA CTG 49
Met Asn Phe Leu Leu Ser Trp Val His Trp Thr Leu Ala Leu Leu
5 10 15

CTG TAC CTC CAC CAT GCC AAG TGG TCC CAG GCT GCA CCC ACG ACA 94
Leu Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Thr Thr
40 20 25 30

GAA GGG GAG CAG AAA GCC CAT GAA GTG GTG AAG TTC ATG GAC GTC 139
Glu Gly Glu Gln Lys Ala His Glu Val Val Lys Phe Met Asp Val
35 40 40

45 TAC CAG CGC AGC TAT TGC CGT CCG ATT GAG ACC CTG GTG GAC ATC 184
Tyr Gln Arg Ser Tyr Cys Arg Pro Ile Glu Thr Leu Val Asp Ile
50 55 60

TTC CAG GAG TAC CCC GAT GAG ATA GAG TAT ATC TTC AAG CCG TCC 229
50 Phe Gln Glu Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser
65 70 75

55

5 TGT GTG CCC CTA ATG CGG TGT GCG GGC TGC TGC AAT GAT GAA GCC 274
 Cys Val Pro Leu Met Arg Cys Ala Gly Cys Cys Asn Asp Glu Ala
 80 85 90
 CTG GAG TGC GTG CCC ACG TCG GAG AGC AAC GTC ACT ATG CAG ATC 319
 Leu Glu Cys Val Pro Thr Ser Glu Ser Asn Val Thr Met Gln Ile
 95 100 105
 10 ATG CGG ATC AAA CCT CAC CAA AGC CAG CAC ATA GGA GAG ATG AGC 364
 Met Arg Ile Lys Pro His Gln Ser Gln His Ile Gly Glu Met Ser
 110 115 120
 TTC CTG CAG CAT AGC AGA TGT GAA TGC AGA CCA AAG AAA GAT AGA 409
 Phe Leu Gln His Ser Arg Cys Glu Cys Arg Pro Lys Lys Asp Arg
 15 125 130 135
 ACA AAG CCA GAA AAT CAC TGT GAG CCT TGT TCA GAG CGG AGA AAG 454
 Thr Lys Pro Glu Asn His Cys Glu Pro Cys Ser Glu Arg Arg Lys
 140 145 150
 20 CAT TTG TTT GTC CAA GAT CCG CAG ACG TGT AAA TGT TCC TGC AAA 499
 His Leu Phe Val Gln Asp Pro Gln Thr Cys Lys Cys Ser Cys Lys
 155 160 165
 AAC ACA GAC TCG CGT TGC AAG GCG AGG CAG CTT GAG TTA AAC GAA 544
 25 Asn Thr Asp Ser Arg Cys Lys Ala Arg Gln Leu Glu Leu Asn Glu
 170 175 180
 CGT ACT TGC AGA TGT GAC AAG CCA AGG CGG TGA 577
 Arg Thr Cys Arg Cys Asp Lys Pro Arg Arg
 30 185 190

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 190 amino acids

(B) TYPE: amino acids

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

40 Met Asn Phe Leu Leu Ser Trp Val His Trp Thr Leu Ala Leu Leu
 5 10 15
 Leu Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Thr Thr
 20 25 30
 45 Glu Gly Glu Gln Lys Ala His Glu Val Val Lys Phe Met Asp Val
 35 40 45
 Tyr Gln Arg Ser Tyr Cys Arg Pro Ile Glu Thr Leu Val Asp Ile
 50 55 60
 50 Phe Gln Glu Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser

55

	65	70	75
5	Cys Val Pro Leu Met Arg Cys Ala Gly Cys Cys Asn Asp Glu Ala 80 85 90		
	Leu Glu Cys Val Pro Thr Ser Glu Ser Asn Val Thr Met Gln Ile 95 100 105		
10	Met Arg Ile Lys Pro His Gln Ser Gln His Ile Gly Glu Met Ser 110 115 120		
	Phe Leu Gln His Ser Arg Cys Glu Cys Arg Pro Lys Lys Asp Arg 125 130 135		
15	Thr Lys Pro Glu Asn His Cys Glu Pro Cys Ser Glu Arg Arg Lys 140 145 150		
	His Leu Phe Val Gln Asp Pro Gln Thr Cys Lys Cys Ser Cys Lys 155 160 165		
20	Asn Thr Asp Ser Arg Cys Lys Ala Arg Gln Leu Glu Leu Asn Glu 170 175 180		
	Arg Thr Cys Arg Cys Asp Lys Pro Arg Arg 185 190		

- (2) INFORMATION FOR SEQ ID NO:32:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 445 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

ACCA 4

35 ATG AAC TTT CTG CTC TCT TGG GTG CAC TGG ACC CTG GCT TTA CTG 49
Met Asn Phe Leu Leu Ser Trp Val His Trp Thr Leu Ala Leu Leu
5 10 15

40 CTG TAC CTC CAC CAT GCC AAG TGG TCC CAG GCT GCA CCC ACG ACA 94
Leu Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Thr Thr
20 25 30

GAA GGG GAG CAG AAA GCC CAT GAA GTG GTG AAG TTC ATG GAC GTC 139
Glu Gly Glu Gln Lys Ala His Glu Val Val Lys Phe Met Asp Val
35 40 45

45 TAC CAG CGC AGC TAT TGC CGT CCG ATT GAG ACC CTG GTG GAC ATC 184
Tyr Gln Arg Ser Tyr Cys Arg Pro Ile Glu Thr Leu Val Asp Ile
50 55 60

50 TTC CAG GAG TAC CCC GAT GAG ATA GAG TAT ATC TTC AAG CCG TCC 229
Phe Gln Glu Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser

	65	70	75	
5	TGT GTG CCC CTA ATG CGG TGT GCG GGC TGC TGC AAT GAT GAA GCC	274		
	Cys Val Pro Leu Met Arg Cys Ala Gly Cys Cys Asn Asp Glu Ala			
	80	85	90	
10	CTG GAG TGC GTG CCC ACG TCG GAG AGC AAC GTC ACT ATG CAG ATC	319		
	Leu Glu Cys Val Pro Thr Ser Glu Ser Asn Val Thr Met Gln Ile			
	95	100	105	
15	ATG CGG ATC AAA CCT CAC CAA AGC CAG CAC ATA GGA GAG ATG AGC	364		
	Met Arg Ile Lys Pro His Gln Ser Gln His Ile Gly Glu Met Ser			
	110	115	120	
20	TTC CTG CAG CAT AGC AGA TGT GAA TGC AGA CCA AAG AAA GAT AGA	409		
	Phe Leu Gln His Ser Arg Cys Glu Cys Arg Pro Lys Lys Asp Arg			
	125	130	135	
25	ACA AAG CCA GAA AAA TGT GAC AAG CCA AGG CGG TGA	445		
	Thr Lys Pro Glu Lys Cys Asp Lys Pro Arg Arg			
	140	145		

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 146 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

30	Met Asn Phe Leu Leu Ser Trp Val His Trp Thr Leu Ala Leu Leu	5	10	15
	Leu Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Thr Thr	20	25	30
35	Glu Gly Glu Gln Lys Ala His Glu Val Val Lys Phe Met Asp Val	35	40	45
	Tyr Gln Arg Ser Tyr Cys Arg Pro Ile Glu Thr Leu Val Asp Ile	50	55	60
40	Phe Gln Glu Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser	65	70	75
	Cys Val Pro Leu Met Arg Cys Ala Gly Cys Cys Asn Asp Glu Ala	80	85	90
45	Leu Glu Cys Val Pro Thr Ser Glu Ser Asn Val Thr Met Gln Ile	95	100	105
50	Met Arg Ile Lys Pro His Gln Ser Gln His Ile Gly Glu Met Ser	110	115	120

55

Phe Leu Gln His Ser Arg Cys Glu Cys Arg Pro Lys Lys Asp Arg
 125 130 135

5 Thr Lys Pro Glu Lys Cys Asp Lys Pro Arg Arg
 140 145

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

10 (A) LENGTH: 649 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (xi) SEQUENCE DESCRIPTION: SEQ
 15 ID NO:34:

AACC

4

20 ATG AAC TTT CTG CTC TCT TGG GTG CAC TGG ACC CTG GCT TTA CTG 49
 Met Asn Phe Leu Leu Ser Trp Val His Trp Thr Leu Ala Leu Leu
 5 10 15

CTG TAC CTC CAC CAT GCC AAG TGG TCC CAG GCT GCA CCC ACG ACA 94
 Leu Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Thr Thr
 20 25 30

25 GAA GGG GAG CAG AAA GCC CAT GAA GTG GTG AAG TTC ATG GAC GTC 139
 Glu Gly Glu Gln Lys Ala His Glu Val Val Lys Phe Met Asp Val
 35 40 45

30 TAC CAG CGC AGC TAT TGC CGT CCG ATT GAG ACC CTG GTG GAC ATC 184
 Tyr Gln Arg Ser Tyr Cys Arg Pro Ile Glu Thr Leu Val Asp Ile
 50 55 60

35 TTC CAG GAG TAC CCC GAT GAG ATA GAG TAT ATC TTC AAG CCG TCC 229
 Phe Gln Glu Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser
 65 70 75

TGT GTG CCC CTA ATG CGG TGT GCG GGC TGC TGC AAT GAT GAA GCC 274
 Cys Val Pro Leu Met Arg Cys Ala Gly Cys Cys Asn Asp Glu Ala
 80 85 90

40 CTG GAG TGC GTG CCC ACG TCG GAG AGC AAC GTC ACT ATG CAG ATC 319
 Leu Glu Cys Val Pro Thr Ser Glu Ser Asn Val Thr Met Gln Ile
 95 100 105

45 ATG CGG ATC AAA CCT CAC CAA AGC CAG CAC ATA GGA GAG ATG AGC 364
 Met Arg Ile Lys Pro His Gln Ser Gln His Ile Gly Glu Met Ser
 110 115 120

TTC CTG CAG CAT AGC AGA TGT GAA TGC AGA CCA AAG AAA GAT AGA 409
 Phe Leu Gln His Ser Arg Cys Glu Cys Arg Pro Lys Lys Asp Arg
 125 130 135

50 ACA AAG CCA GAA AAA AAA TCA GTT CGA GGA AAG GGA AAG GGT CAA 454

55

Thr Lys Pro Glu Lys Lys Ser Val Arg Gly Lys Gly Lys Gly Gln
 140 145 150
 5 AAA CGA AAG CGC AAG AAA TCC CGG TTT AAA TCC TGG AGC GTT CAC 499
 Lys Arg Lys Arg Lys Lys Ser Arg Phe Lys Ser Trp Ser Val His
 155 160 165
 TGT GAG CCT TGT TCA GAG CGG AGA AAG CAT TTG TTT GTC CAA GAT 544
 10 Cys Glu Pro Cys Ser Glu Arg Arg Lys His Leu Phe Val Gln Asp
 170 175 180
 CCG CAG ACG TGT AAA TGT TCC TGC AAA AAC ACA GAC TCG CGT TGC 589
 Pro Gln Thr Cys Lys Cys Ser Cys Lys Asn Thr Asp Ser Arg Cys
 185 190 195
 15 AAG GCG AGG CAG CTT GAG TTA AAC GAA CGT ACT TGC AGA TGT GAC 634
 Lys Ala Arg Gln Leu Glu Leu Asn Glu Arg Thr Cys Arg Cys Asp
 200 205 210
 AAG CCA AGG CGG TGA 649
 20 Lys Pro Arg Arg

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:

25 (A) LENGTH: 214 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

30 Met Asn Phe Leu Leu Ser Trp Val His Trp Thr Leu Ala Leu Leu
 5 10 15
 Leu Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Thr Thr
 20 25 30
 35 Glu Gly Glu Gln Lys Ala His Glu Val Val Lys Phe Met Asp Val
 35 40 45
 Tyr Gln Arg Ser Tyr Cys Arg Pro Ile Glu Thr Leu Val Asp Ile
 50 55 60
 40 Phe Gln Glu Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser
 65 70 75
 Cys Val Pro Leu Met Arg Cys Ala Gly Cys Cys Asn Asp Glu Ala
 80 85 90
 45 Leu Glu Cys Val Pro Thr Ser Glu Ser Asn Val Thr Met Gln Ile
 95 100 105
 Met Arg Ile Lys Pro His Gln Ser Gln His Ile Gly Glu Met Ser
 110 115 120
 50
 55

Phe Leu Gln His Ser Arg Cys Glu Cys Arg Pro Lys Lys Asp Arg
 125 130 135
 5 Thr Lys Pro Glu Lys Lys Ser Val Arg Gly Lys Gly Lys Gly Gln
 140 145 150
 Lys Arg Lys Arg Lys Lys Ser Arg Phe Lys Ser Trp Ser Val His
 155 160 165
 10 Cys Glu Pro Cys Ser Glu Arg Arg Lys His Leu Phe Val Gln Asp
 170 175 180
 Pro Gln Thr Cys Lys Cys Ser Cys Lys Asn Thr Asp Ser Arg Cys
 185 190 195
 15 Lys Ala Arg Gln Leu Glu Leu Asn Glu Arg Thr Cys Arg Cys Asp
 200 205 210
 Lys Pro Arg Arg

(2) INFORMATION FOR SEQ ID NO:36:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 417 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

ATG CTG GCC ATG AAG CTG TTC ACT TGC TTC TTG CAG GTC CTA GCT 45
 Met Leu Ala Met Lys Leu Phe Thr Cys Phe Leu Gln Val Leu Ala 15
 30 GGG TTG GCT GTG CAC TCC CAG GGG GCC CTG TCT GCT GGG AAC AAC 90
 Gly Leu Ala Val His Ser Gln Gly Ala Leu Ser Ala Gly Asn Asn 30
 35 TCA ACA GAA ATG GAA GTG GTG CCT TTC AAT GAA GTG TGG GGC CGC 135
 Ser Thr Glu Met Glu Val Val Pro Phe Asn Glu Val Trp Gly Arg 45
 40 AGC TAC TGC CGG CCA ATG GAG AAG CTG GTG TAC ATT GCA GAT GAA 180
 Ser Tyr Cys Arg Pro Met Glu Lys Leu Val Tyr Ile Ala Asp Glu 60
 CAC CCT AAT GAA GTG TCT CAT ATA TTC AGT CCG TCA TGT GTC CTT 225
 His Pro Asn Glu Val Ser His Ile Phe Ser Pro Ser Cys Val Leu 75
 45 CTG AGT CGC TGT AGT GGC TGC TGT GGT GAC GAG GGT CTG CAC TGT 270
 Leu Ser Arg Cys Ser Gly Cys Cys Gly Asp Glu Gly Leu His Cys 90
 80 85 90

50

55

GTG GCG CTA AAG ACA GCC AAC ATC ACT ATG CAG ATC TTA AAG ATT 315
 Val Ala Leu Lys Thr Ala Asn Ile Thr Met Gln Ile Leu Lys Ile
 95 100 105

5 CCC CCC AAT CGG GAT CCA CAT TCC TAC GTG GAG ATG ACA TTC TCT 360
 Pro Pro Asn Arg Asp Pro His Ser Tyr Val Glu Met Thr Phe Ser
 110 115 120

10 CAG GAT GTA CTC TGC GAA TGC AGG CCT ATT CTG GAG ACG ACA AAG 405
 Gln Asp Val Leu Cys Glu Cys Arg Pro Ile Leu Glu Thr Thr Lys
 125 130 135

GCA GAA AGG TAA 417
 Ala Glu Arg

15 (2) INFORMATION FOR SEQ ID NO:37:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 138 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

Met Leu Ala Met Lys Leu Phe Thr Cys Phe Leu Gln Val Leu Ala
 5 10 15
 25 Gly Leu Ala Val His Ser Gln Gly Ala Leu Ser Ala Gly Asn Asn
 20 25 30
 Ser Thr Glu Met Glu Val Val Pro Phe Asn Glu Val Trp Gly Arg
 35 40 45
 30 Ser Tyr Cys Arg Pro Met Glu Lys Leu Val Tyr Ile Ala Asp Glu
 50 55 60
 His Pro Asn Glu Val Ser His Ile Phe Ser Pro Ser Cys Val Leu
 65 70 75
 35 Leu Ser Arg Cys Ser Gly Cys Cys Gly Asp Glu Gly Leu His Cys
 80 85 90
 Val Ala Leu Lys Thr Ala Asn Ile Thr Met Gln Ile Leu Lys Ile
 95 100 105
 40 Pro Pro Asn Arg Asp Pro His Ser Tyr Val Glu Met Thr Phe Ser
 110 115 120
 45 Gln Asp Val Leu Cys Glu Cys Arg Pro Ile Leu Glu Thr Thr Lys
 125 130 135
 Ala Glu Arg

50 (2) INFORMATION FOR SEQ ID NO:38:
 (i) SEQUENCE CHARACTERISTICS:

55

(A) LENGTH: 477 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

ATG CTG GCC ATG AAG CTG TTC ACT TGC TTC TTG CAG GTC CTA GCT	45
Met Leu Ala Met Lys Leu Phe Thr Cys Phe Leu Gln Val Leu Ala	
5 10 15	
GGG TTG GCT GTG CAC TCC CAG GGG GCC CTG TCT GCT GGG AAC AAC	90
Gly Leu Ala Val His Ser Gln Gly Ala Leu Ser Ala Gly Asn Asn	
20 25 30	
TCA ACA GAA ATG GAA GTG GTG CCT TTC AAT GAA GTG TGG GGC CGC	135
Ser Thr Glu Met Glu Val Val Pro Phe Asn Glu Val Trp Gly Arg	
35 40 45	
AGC TAC TGC CGG CCA ATG GAG AAG CTG GTG TAC ATT GCA GAT GAA	180
Ser Tyr Cys Arg Pro Met Glu Lys Leu Val Tyr Ile Ala Asp Glu	
50 55 60	
CAC CCT AAT GAA GTG TCT CAT ATA TTC AGT CCG TCA TGT GTC CTT	225
His Pro Asn Glu Val Ser His Ile Phe Ser Pro Ser Cys Val Leu	
65 70 75	
CTG AGT CGC TGT AGT GGC TGC TGT GGT GAC GAG GGT CTG CAC TGT	270
Leu Ser Arg Cys Ser Gly Cys Cys Gly Asp Glu Gly Leu His Cys	
80 85 90	
GTG GCG CTA AAG ACA GCC AAC ATC ACT ATG CAG ATC TTA AAG ATT	315
Val Ala Leu Lys Thr Ala Asn Ile Thr Met Gln Ile Leu Lys Ile	
95 100 105	
CCC CCC AAT CGG GAT CCA CAT TCC TAC GTG GAG ATG ACA TTC TCT	360
Pro Pro Asn Arg Asp Pro His Ser Tyr Val Glu Met Thr Phe Ser	
110 115 120	
CAG GAT GTA CTC TGC GAA TGC AGG CCT ATT CTG GAG ACG ACA AAG	405
Gln Asp Val Leu Cys Glu Cys Arg Pro Ile Leu Glu Thr Thr Lys	
125 130 135	
GCA GAA AGG AGG AAA ACC AAG GGG AAG AGG AAG CAA AGC AAA ACC	450
Ala Glu Arg Arg Lys Thr Lys Gly Lys Arg Lys Gln Ser Lys Thr	
140 145 150	
CCA CAG ACT GAG GAA CCC CAC CTG TGA	477
Pro Gln Thr Glu Glu Pro His Leu	
155	

(2) INFORMATION FOR SEQ ID NO:39:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 158 amino acids

(B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

5

Met Leu Ala Met Lys Leu Phe Thr Cys Phe Leu Gln Val Leu Ala
 5 10 15

10

Gly Leu Ala Val His Ser Gln Gly Ala Leu Ser Ala Gly Asn Asn
 20 25 30

Ser Thr Glu Met Glu Val Val Pro Phe Asn Glu Val Trp Gly Arg
 35 40 45

15

Ser Tyr Cys Arg Pro Met Glu Lys Leu Val Tyr Ile Ala Asp Glu
 50 55 60

His Pro Asn Glu Val Ser His Ile Phe Ser Pro Ser Cys Val Leu
 65 70 75

20

Leu Ser Arg Cys Ser Gly Cys Cys Gly Asp Glu Gly Leu His Cys
 80 85 90

Val Ala Leu Lys Thr Ala Asn Ile Thr Met Gln Ile Leu Lys Ile
 95 100 105

25

Pro Pro Asn Arg Asp Pro His Ser Tyr Val Glu Met Thr Phe Ser
 110 115 120

Gln Asp Val Leu Cys Glu Cys Arg Pro Ile Leu Glu Thr Thr Lys
 125 130 135

30

Ala Glu Arg Arg Lys Thr Lys Gly Lys Arg Lys Gln Ser Lys Thr
 140 145 150

Pro Gln Thr Glu Glu Pro His Leu
 155

35

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 465 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

45

ATG CCG GTC ATG AGG CTG TTC CCT TGC TTC CTG CAG CTC CTG GCC 45
 Met Pro Val Met Arg Leu Phe Pro Cys Phe Leu Gln Leu Leu Ala
 5 10 15

50

55

EP 0 506 477 A1

GGG CTG GCG CTG CCT GCT GTG CCC CCC CAG CAG TGG GCC TTG TCT 90
 Gly Leu Ala Leu Pro Ala Val Pro Pro Gln Gln Trp Ala Leu Ser
 20 25 30

5 GCT GGG AAC GGC TCG TCA GAG GTG GAA GTG GTA CCC TTC CAG GAA 135
 Ala Gly Asn Gly Ser Ser Glu Val Glu Val Val Pro Phe Gln Glu
 35 40 45

10 GTG TGG GGC CGC AGC TAC TGC CGG GCG CTG GAG AGG CTG GTG GAC 180
 Val Trp Gly Arg Ser Tyr Cys Arg Ala Leu Glu Arg Leu Val Asp
 50 55 60

15 GTC GTG TCC GAG TAC CCC AGC GAG GTG GAG CAC ATG TTC AGC CCA 225
 Val Val Ser Glu Tyr Pro Ser Glu Val Glu His Met Phe Ser Pro
 65 70 75

TCC TGT GTC TCC CTG CTG CGC TGC ACC GGC TGC TGC GGC GAT GAG 270
 Ser Cys Val Ser Leu Leu Arg Cys Thr Gly Cys Cys Gly Asp Glu
 80 85 90

20 AAT CTG CAC TGT GTG CCG GTG GAG ACG GCC AAT GTC ACC ATG CAG 315
 Asn Leu His Cys Val Pro Val Glu Thr Ala Asn Val Thr Met Gln
 95 100 105

25 CTC CTA AAG ATC CGT TCT GGG GAC CGG CCC TCC TAC GTG GAG CTG 360
 Leu Leu Lys Ile Arg Ser Gly Asp Arg Pro Ser Tyr Val Glu Leu
 110 115 120

ACG TTC TCT CAG CAC GTT CGC TGC GAA TGC CGG CCT CTG CGG GAG 405
 Thr Phe Ser Gln His Val Arg Cys Glu Cys Arg Pro Leu Arg Glu
 125 130 135

30 AAG ATG AAG CCG GAA AGG AGG AGA CCC AAG GGC AGG GGG AAG AGG 450
 Lys Met Lys Pro Glu Arg Arg Arg Pro Lys Gly Arg Gly Lys Arg
 140 145 150

35 AGG AGA GAG AAG TAG 465
 Arg Arg Glu Lys

(2) INFORMATION FOR SEQ ID NO:41:

40 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 154 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

45 Met Pro Val Met Arg Leu Phe Pro Cys Phe Leu Gln Leu Leu Ala
 5 10 15

50 Gly Leu Ala Leu Pro Ala Val Pro Pro Gln Gln Trp Ala Leu Ser
 20 25 30

55

[illegible]

Claims

1. A purified and isolated DNA sequence encoding the C subunit of vascular endothelial cell growth factor.
2. A purified and isolated vascular endothelial cell growth factor C subunit DNA sequence comprising:

35 ATG CCG GTC ATG AGG CTG TTC OCT TGC TTC CTG CAG CTC CTG
GOC GGG CTG GOG CTG OCT GCT GTG OCC OCC CAG CAG TGG GCC
TTG TCT GCT GGG AAC GGC TOG TCA GAG GTG GAA GTG GTA CCC
TTC CAG GAA GTG TGG GGC OGC AGC TAC TGC OGG GCG CTG GAG
40 AGG CTG GTG GAC GTC GTG TOC GAG TAC OCC AGC GAG GTG GAG
CAC ATG TTC AGC CCA TOC TGT GTC TOC CTG CTG OGC TGC ACC
GGC TGC TGC GGC GAT GAG AAT CTG CAC TGT GTG OCG GTG GAG
45 ACG GOC AAT GTC ACC ATG CAG CTC CTA AAG ATC OGT TCT GGG
GAC OGG OCC TOC TAC GTG GAG CTG ACG TTC TCT CAG CAC GTT
OGC TGC GAA TGC OGG OCT CTG OGG GAG AAG ATG AAG CCG GAA
AGG AGG AGA OCC AAG GGC AGG GGG AAG AGG AGG AGA GAG AAG
50 TAG. SEQ ID NO:40

- 55
3. Vascular endothelial cell growth growth factor AC DNA comprising an A subunit DNA sequence and a C subunit DNA sequence.
 4. Vascular endothelial cell growth growth factor BC DNA comprising a B subunit DNA sequence and a C subunit DNA sequence.

5. A purified and isolated vascular endothelial cell growth factor C subunit DNA sequence comprising:

ATG CCG GTC ATG AGG CTG TTC OCT TGC TTC CTG CAG CTC CTG

5 GGC GGG CTG GCG CTG CCT GCT GTG CCG CCG CAG CAG TGG GCC
 TTG TCT GCT GGG AAC GGC TCG TCA GAG GTG GAA GTG GTA CCC
 TTC CAG GAA GTG TGG GGC OGC AGC TAC TGC OGG GCG CTG GAG
 10 AGG CTG GTG GAC GTC GTG TOC GAG TAC CCG AGC GAG GTG GAG
 CAC ATG TTC AGC OCA TOC TGT GTC TOC CTG CTG CCG TGC ACC
 GGC TGC TGC GGC GAT GAG AAT CTG CAC TGT GTG CCG GTG GAG
 ACG GCC AAT GTC ACC ATG CAG CTC CTA AAG ATC CGT TCT GGG
 15 GAC OGG CCC TOC TAC GTG GAG CTG ACG TTC TCT CAG CAC GTT
 CCG TGC GAA TGC CCG CCT CTG OGG GAG AAG ATG AAG CCG GAA
 AGG AGG AGA CCG AAG GGC AGG GGG AAG AGG AGG AGA GAG AAG
 20 CAG AGA CCC ACA GAC TGC CAC CTG TGC GGC GAT GCT GTT CCC
 CCG AGG TAA. SEQ ID NOS:29 & 40

6. Vascular endothelial cell growth factor AC DNA comprising an A subunit DNA sequence selected from the group consisting of: a DNA sequence encoding an 189 amino acid form, a DNA sequence encoding an 165 amino acid form and a DNA sequence encoding a 121 amino acid form, with said A subunit DNA operably attached to a C subunit DNA sequence.
7. Vascular endothelial cell growth factor BC DNA comprising a B subunit DNA sequence selected from the group consisting of a DNA sequence encoding a 135 amino acid form; and a DNA sequence encoding a 115 amino acid form, with said B subunit DNA sequence operably attached to a C subunit DNA sequence.
8. Homodimeric vascular endothelial growth factor DNA comprising C subunit DNA sequences.
9. A vector containing the DNA sequence of any one of claims 3 to 8.
10. A host cell transformed by the vector of claim 9 containing the DNA sequence encoding vascular endothelial cell growth factor.
11. A process for the preparation of vascular endothelial cell growth factor comprising culturing the transformed host cell of claim 10 under conditions suitable for the expression of vascular endothelial cell growth factor and recovering vascular endothelial cell growth factor.
12. Vascular endothelial growth factor made by the process of claim 11.
13. Vascular endothelial cell growth factor AC comprising an A subunit amino acid sequence and a C subunit amino acid sequence.
14. Vascular endothelial cell growth factor BC comprising a B subunit amino acid sequence and a C subunit amino acid sequence.
15. Vascular endothelial cell growth factor CC comprising a C subunit amino acid sequence and a C subunit amino acid sequence.
16. A purified and isolated vascular endothelial cell growth factor C subunit amino acid sequence comprising:

Met Pro Val Met Arg Leu Phe Pro Cys Phe Leu Gln Leu Leu
 Ala Gly Leu Ala Leu Pro Ala Val Pro Pro Gln Gln Trp Ala
 5 Leu Ser Ala Gly Asn Gly Ser Ser Glu Val Glu Val Val Pro
 Phe Gln Glu Val Trp Gly Arg Ser Tyr Cys Arg Ala Leu Glu
 Arg Leu Val Asp Val Val Ser Glu Tyr Pro Ser Glu Val Glu
 His Met Phe Ser Pro Ser Cys Val Ser Leu Leu Arg Cys Thr
 10 Gly Cys Cys Gly Asp Glu Asn Leu His Cys Val Pro Val Glu
 Thr Ala Asn Val Thr Met Gln Leu Leu Lys Ile Arg Ser Gly
 Asp Arg Pro Ser Tyr Val Glu Leu Thr Phe Ser Gln His Val
 15 Arg Cys Glu Cys Arg Pro Leu Arg Glu Lys Met Lys Pro Glu
 Arg Arg Arg Pro Lys Gly Arg Gly Lys Arg Arg Arg Glu Lys.
 SEQ ID NO: 41

20 17. A purified and isolated vascular endothelial cell growth factor C subunit amino acid sequence comprising:

Met Pro Val Met Arg Leu Phe Pro Cys Phe Leu Gln Leu Leu
 25 Ala Gly Leu Ala Leu Pro Ala Val Pro Pro Gln Gln Trp Ala
 Leu Ser Ala Gly Asn Gly Ser Ser Glu Val Glu Val Val Pro
 Phe Gln Glu Val Trp Gly Arg Ser Tyr Cys Arg Ala Leu Glu
 Arg Leu Val Asp Val Val Ser Glu Tyr Pro Ser Glu Val Glu
 30 His Met Phe Ser Pro Ser Cys Val Ser Leu Leu Arg Cys Thr
 Gly Cys Cys Gly Asp Glu Asn Leu His Cys Val Pro Val Glu
 Thr Ala Asn Val Thr Met Gln Leu Leu Lys Ile Arg Ser Gly
 Asp Arg Pro Ser Tyr Val Glu Leu Thr Phe Ser Gln His Val
 35 Arg Cys Glu Cys Arg Pro Leu Arg Glu Lys Met Lys Pro Glu
 Arg Arg Arg Pro Lys Gly Arg Gly Lys Arg Arg Arg Glu Lys
 Gln Arg Pro Thr Asp Cys His Leu Cys Gly Asp Ala Val Pro
 40 Arg Arg. SEQ ID NOS: 29 & 40

18. A tissue repairing pharmaceutical composition comprising a pharmaceutical carrier and an effective tissue repairing amount of the purified vascular endothelial growth factor of any one of claims 13 to 15,

45 19. The use of the vascular endothelial cell growth factor of any one of claims 13 to 15 for the manufacture of a medicament for promoting tissue repair.

20. The use of the vascular endothelial cell growth factor of any one of claims 13 to 15 for the manufacture of a medicament for stimulating vascular endothelial cell growth.

55

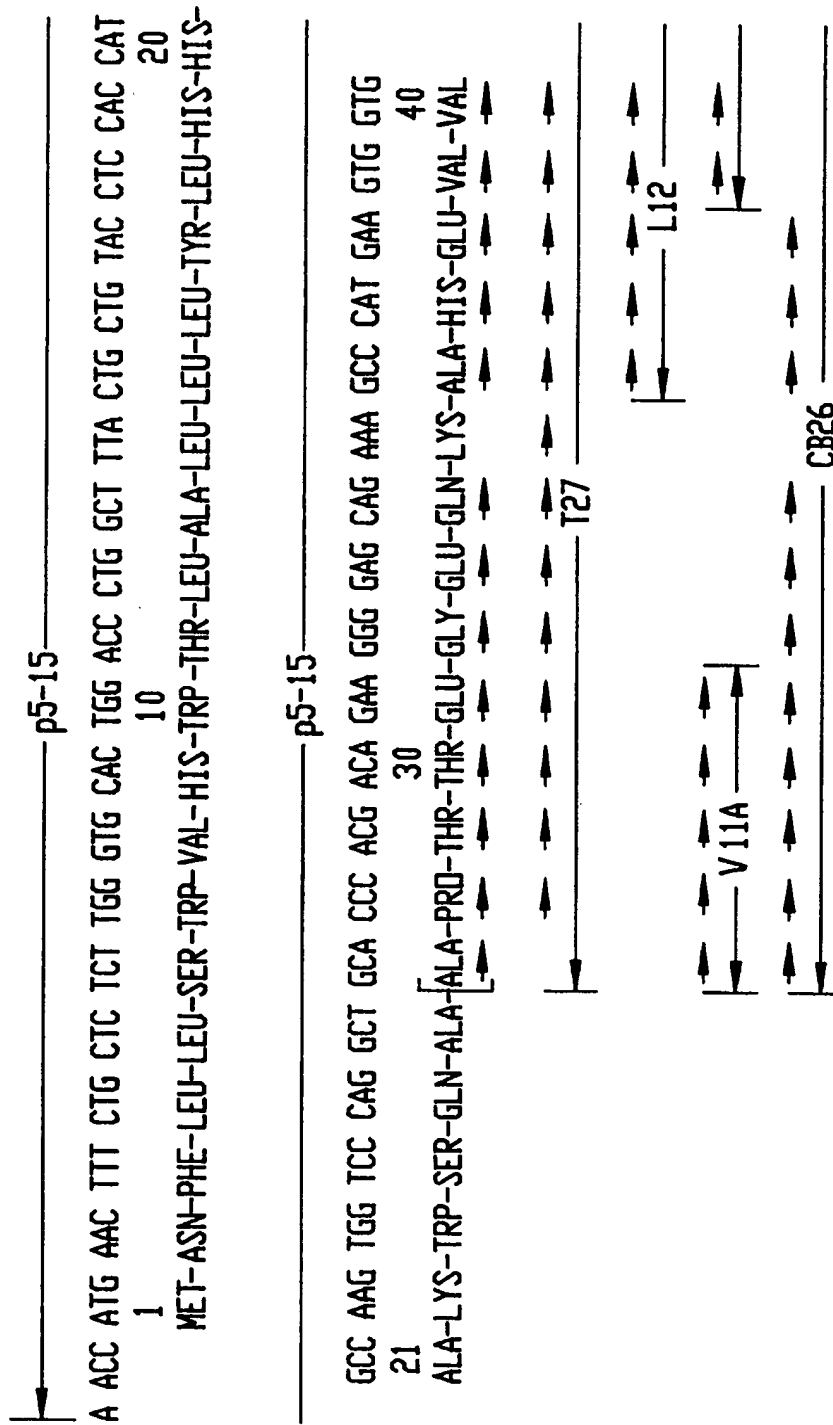


FIG.1A

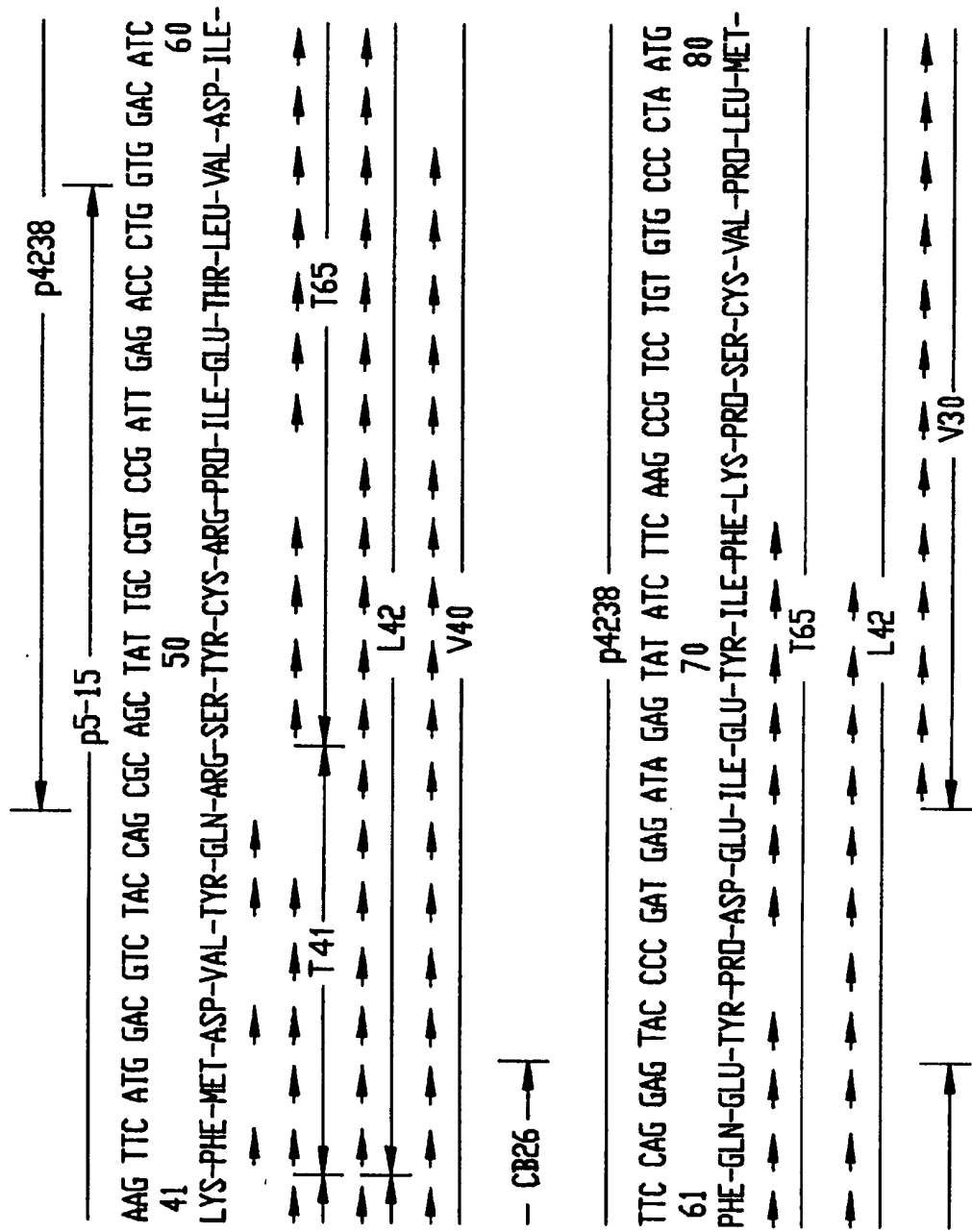


FIG.1B

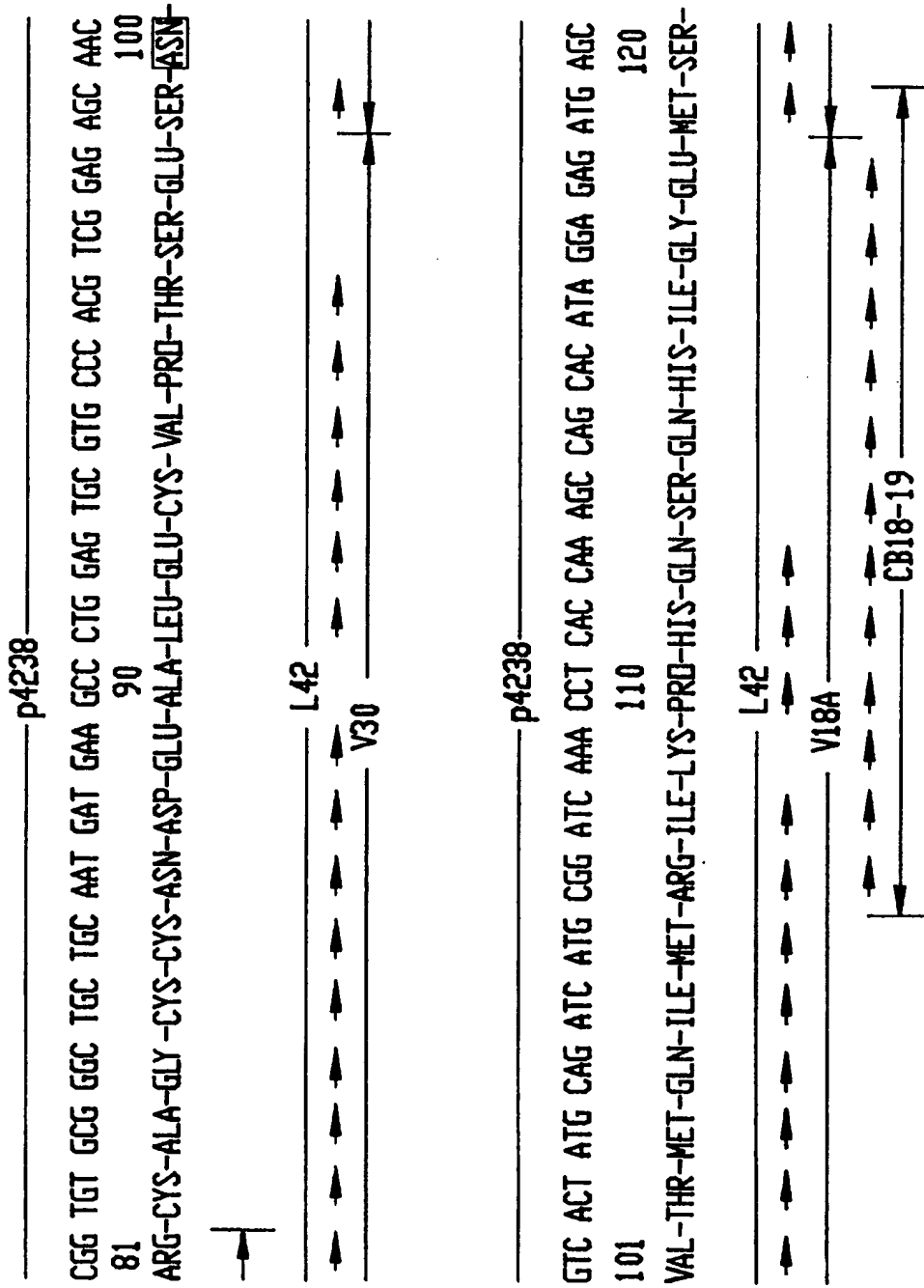


FIG.1C

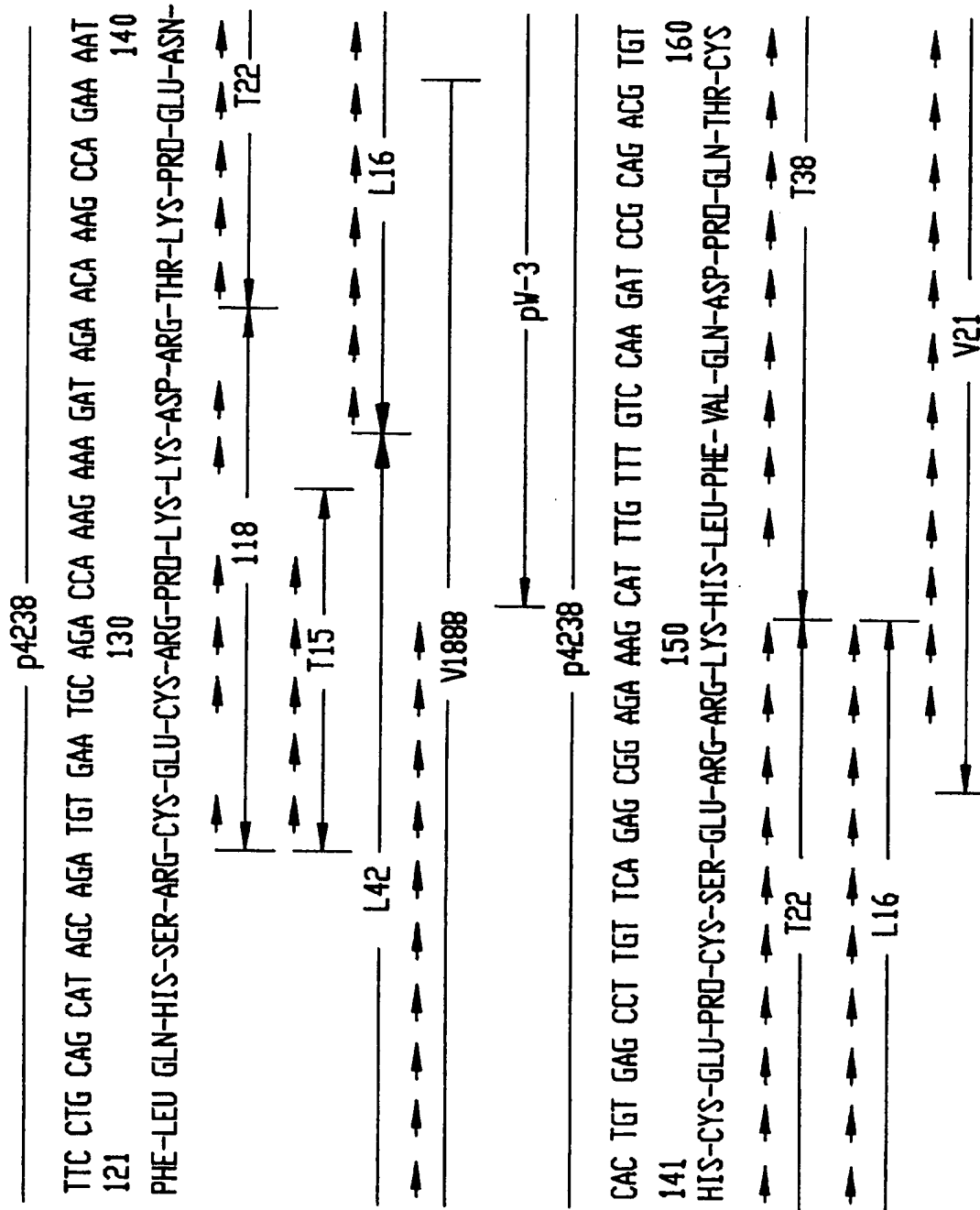


FIG.1D

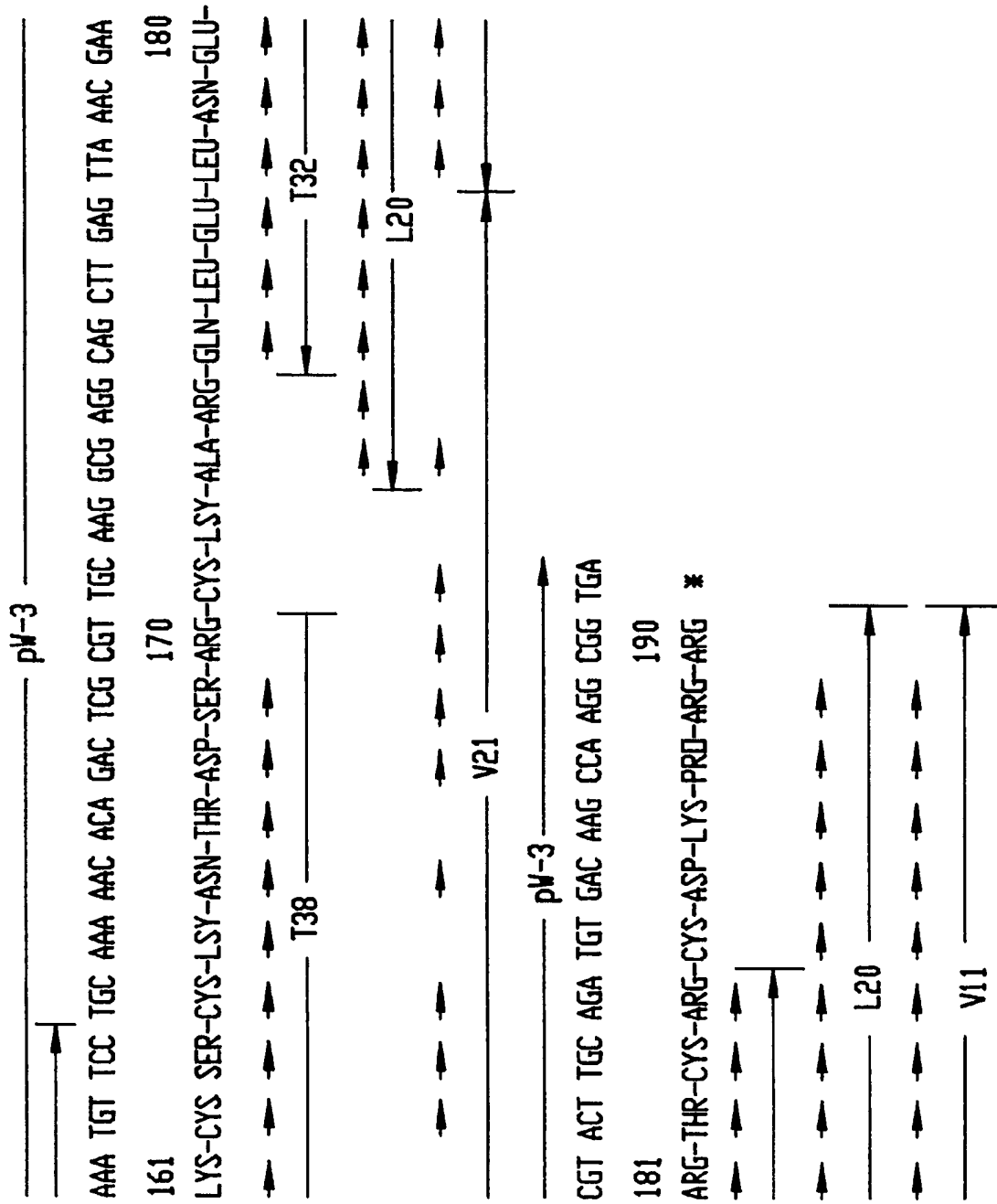


FIG.1E

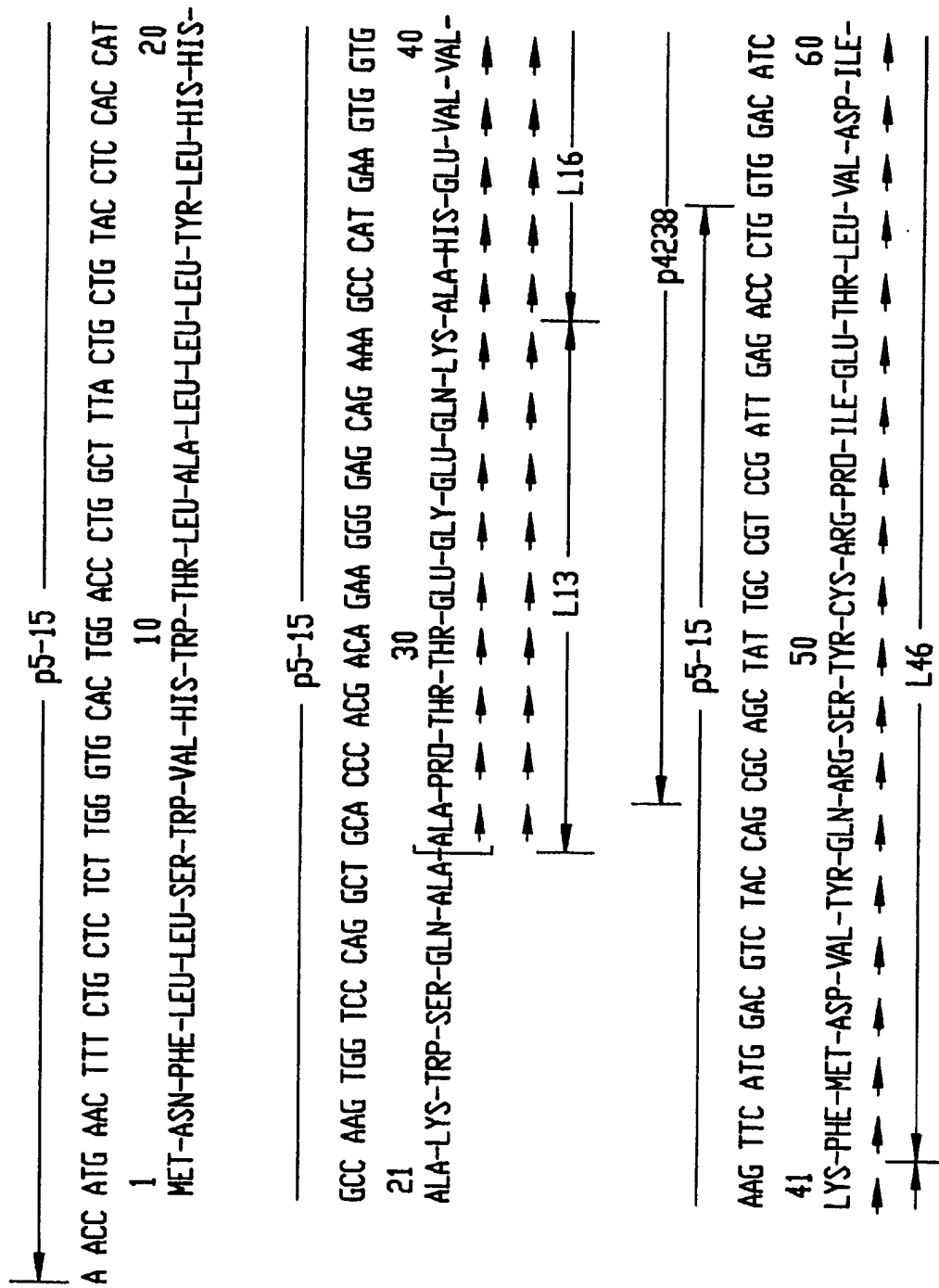


FIG.2A

_____ p4238 _____
 GTC ACT ATG CAG ATC ATG CGG ATC AAA CCT CAC CAG CAG CAC ATA GGA GAG ATG AGC
 101 110 120
 VAL - THR - MET - GLN - ILE - MET - ARG - ILE - LYS - PRO - HIS - GLN - SER - GLN - HIS - ILE - GLY - GLU - MET - SER -
 _____ L46 _____

FIG. 2B

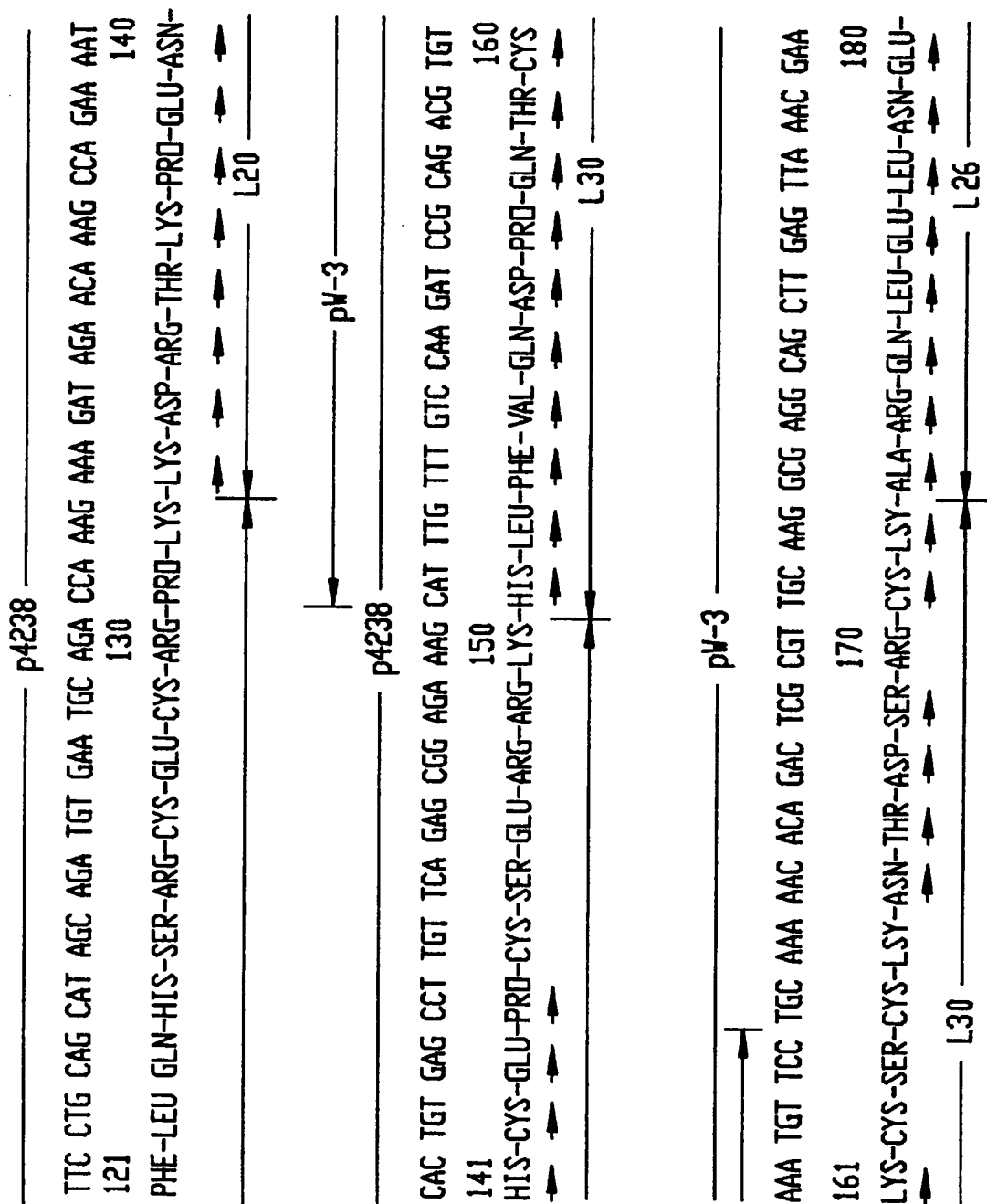


FIG.2C

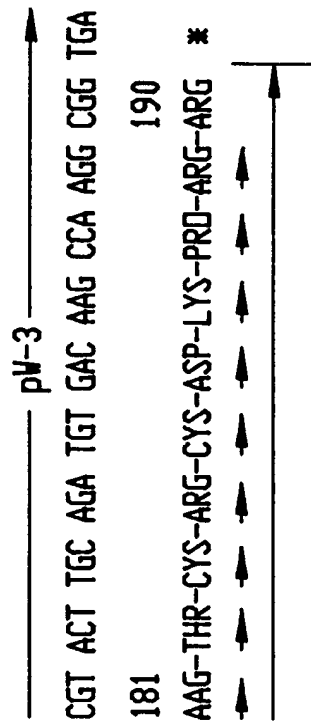


FIG. 2D

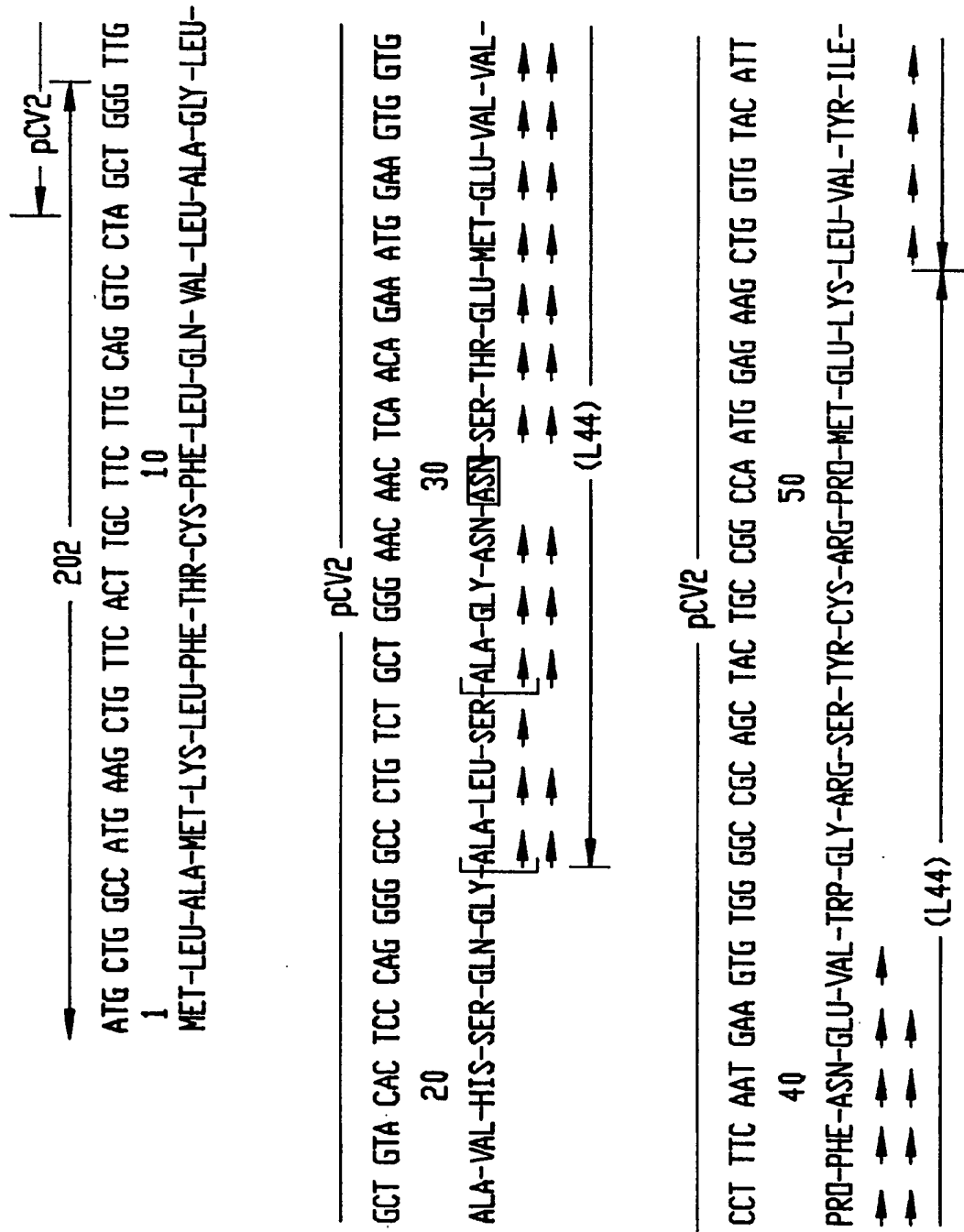


FIG.3A

FIG. 3B

ACCA ATG AAC TTT CTG CTC TCT TGG GTG CAC TGG ACC CTG GCT TTA CTG	49
Met Asn Phe Leu Leu Ser Trp Val His Trp Thr Leu Ala Leu Leu	
5 10 15	
CTG TAC CTC CAC CAT GOC AAG TGG TOC CAG GCT GCA OOC ACG ACA	94
Leu Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Thr Thr	
20 25 30	
GAA GGG GAG CAG AAA GOC CAT GAA GTG GTG AAG TTC ATG GAC GTC	139
Glu Gly Glu Gln Lys Ala His Glu Val Val Lys Phe Met Asp Val	
35 40 45	
TAC CAG CGC AGC TAT TGC CGT CCG ATT GAG ACC CTG GTG GAC ATC	184
Tyr Gln Arg Ser Tyr Cys Arg Pro Ile Glu Thr Leu Val Asp Ile	
50 55 60	
TTC CAG GAG TAC CCC GAT GAG ATA GAG TAT ATC TTC AAG CCG TOC	229
Phe Gln Glu Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser	
65 70 75	
TGT GTG CCC CTA ATG CCG TGT GCG GGC TGC TGC AAT GAT GAA GOC	274
Cys Val Pro Leu Met Arg Cys Ala Gly Cys Cys Asn Asp Glu Ala	
80 85 90	
CTG GAG TGC GTG CCC ACG TCG GAG AGC AAC GTC ACT ATG CAG ATC	319
Leu Glu Cys Val Pro Thr Ser Glu Ser Asn Val Thr Met Gln Ile	
95 100 105	
ATG CCG ATC AAA CCT CAC CAA AGC CAG CAC ATA GGA GAG ATG AGC	364
Met Arg Ile Lys Pro His Gln Ser Gln His Ile Gly Glu Met Ser	
110 115 120	
TTC CTG CAG CAT AGC AGA TGT GAA TGC AGA CCA AAG AAA GAT AGA	409
Phe Leu Gln His Ser Arg Cys Glu Cys Arg Pro Lys Lys Asp Arg	
125 130 135	
ACA AAG CCA GAA AAA TGT GAC AAG CCA AGG CCG TGA	445
Thr Lys Pro Glu Lys Cys Asp Lys Pro Arg Arg	
140 145	

FIG. 4

AACC ATG AAC TTT CTG CTC TCT TGG GTG CAC TGG ACC CTG GCT TTA CTG	49
Met Asn Phe Leu Leu Ser Trp Val His Trp Thr Leu Ala Leu Leu	
5 10 15	
CTG TAC CTC CAC CAT GGC AAG TGG TOC CAG GCT GCA CCC ACG ACA	94
Leu Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Thr Thr	
20 25 30	
GAA GGG GAG CAG AAA GGC CAT GAA GTG GTG AAG TTC ATG GAC GTC	139
Glu Gly Glu Gln Lys Ala His Glu Val Val Lys Phe Met Asp Val	
35 40 45	
TAC CAG CGC AGC TAT TGC CGT CCG ATT GAG ACC CTG GTG GAC ATC	184
Tyr Gln Arg Ser Tyr Cys Arg Pro Ile Glu Thr Leu Val Asp Ile	
50 55 60	
TTC CAG GAG TAC CCC GAT GAG ATA GAG TAT ATC TTC AAG CCG TOC	229
Phe Gln Glu Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro Ser	
65 70 75	
TGT GTG CCC CTA ATG CCG TGT GCG GGC TGC TGC AAT GAT GAA GGC	274
Cys Val Pro Leu Met Arg Cys Ala Gly Cys Cys Asn Asp Glu Ala	
80 85 90	
CTG GAG TGC GTG CCC ACG TCG GAG AGC AAC GTC ACT ATG CAG ATC	319
Leu Glu Cys Val Pro Thr Ser Glu Ser Asn Val Thr Met Gln Ile	
95 100 105	
ATG CCG ATC AAA CCT CAC CAA AGC CAG CAC ATA GGA GAG ATG AGC	364
Met Arg Ile Lys Pro His Gln Ser Gln His Ile Gly Glu Met Ser	
110 115 120	
TTC CTG CAG CAT AGC AGA TGT GAA TGC AGA CCA AAG AAA GAT AGA	409
Phe Leu Gln His Ser Arg Cys Glu Cys Arg Pro Lys Lys Asp Arg	
125 130 135	
ACA AAG CCA GAA AAT CAC TGT GAG CCT TGT TCA GAG CCG AGA AAG	454
Thr Lys Pro Glu Asn His Cys Glu Pro Cys Ser Glu Arg Arg Lys	
140 145 150	
CAT TTG TTT GTC CAA GAT CCG CAG ACG TGT AAA TGT TOC TGC AAA	499
His Leu Phe Val Gln Asp Pro Gln Thr Cys Lys Cys Ser Cys Lys	
155 160 165	
AAC ACA GAC TOG CGT TGC AAG GCG AGG CAG CTT GAG TTA AAC GAA	544
Asn Thr Asp Ser Arg Cys Lys Ala Arg Gln Leu Glu Leu Asn Glu	
170 175 180	
CGT ACT TGC AGA TGT GAC AAG CCA AGG CCG TGA	577
Arg Thr Cys Arg Cys Asp Lys Pro Arg Arg	
185 190	

FIG. 5

AACC	ATG	AAC	TTT	CTG	CTC	TCT	TGG	GTG	CAC	TGG	AOC	CTG	GCT	TTA	CTG	49
Met	Asn	Phe	Leu	Leu	Ser	Trp	Val	His	Trp	Thr	Leu	Ala	Leu	Leu		
				5						10					15	
CTG	TAC	CTC	CAC	CAT	GOC	AAG	TGG	TOC	CAG	GCT	GCA	OOC	ACG	ACA	94	
Leu	Tyr	Leu	His	His	Ala	Lys	Trp	Ser	Gln	Ala	Ala	Pro	Thr	Thr		
				20					25					30		
GAA	GGG	GAG	CAG	AAA	GOC	CAT	GAA	GTG	GTG	AAG	TTC	ATG	GAC	GTC	139	
Glu	Gly	Glu	Gln	Lys	Ala	His	Glu	Val	Val	Lys	Phe	Met	Asp	Val		
				35					40					45		
TAC	CAG	OGC	AGC	TAT	TGC	OGT	COG	ATT	GAG	AOC	CTG	GTG	GAC	ATC	184	
Tyr	Gln	Arg	Ser	Tyr	Cys	Arg	Pro	Ile	Glu	Thr	Leu	Val	Asp	Ile		
				50					55					60		
TTC	CAG	GAG	TAC	COO	GAT	GAG	ATA	GAG	TAT	ATC	TTC	AAG	COG	TOC	229	
Phe	Gln	Glu	Tyr	Pro	Asp	Glu	Ile	Glu	Tyr	Ile	Phe	Lys	Pro	Ser		
				65					70					75		
TGT	GTG	CCC	CTA	ATG	OGG	TGT	GCG	GGC	TGC	TGC	AAT	GAT	GAA	GOC	274	
Cys	Val	Pro	Leu	Met	Arg	Cys	Ala	Gly	Cys	Cys	Asn	Asp	Glu	Ala		
				80					85					90		
CTG	GAG	TGC	GTG	COO	ACG	TOG	GAG	AGC	AAC	GTC	ACT	ATG	CAG	ATC	319	
Leu	Glu	Cys	Val	Pro	Thr	Ser	Glu	Ser	Asn	Val	Thr	Met	Gln	Ile		
				95					100					105		
ATG	CGG	ATC	AAA	OCT	CAC	CAA	AGC	CAG	CAC	ATA	GGA	GAG	ATG	AGC	364	
Met	Arg	Ile	Lys	Pro	His	Gln	Ser	Gln	His	Ile	Gly	Glu	Met	Ser		
				110					115					120		
TTC	CTG	CAG	CAT	AGC	AGA	TGT	GAA	TGC	AGA	CCA	AAG	AAA	GAT	AGA	409	
Phe	Leu	Gln	His	Ser	Arg	Cys	Glu	Cys	Arg	Pro	Lys	Lys	Asp	Arg		
				125					130					135		
ACA	AAG	CCA	GAA	AAA	AAA	TCA	GTT	OGA	GGA	AAG	GGA	AAG	GGT	CAA	454	
Thr	Lys	Pro	Glu	Lys	Lys	Ser	Val	Arg	Gly	Lys	Gly	Lys	Gly	Gln		
				140					145					150		
AAA	CGA	AAG	OGC	AAG	AAA	TOC	CGG	TTT	AAA	TOC	TGG	AGC	GTT	CAC	499	
Lys	Arg	Lys	Arg	Lys	Lys	Ser	Arg	Phe	Lys	Ser	Trp	Ser	Val	His		
				155					160					165		

FIG. 6A

TGT	GAG	OCT	TGT	TCA	GAG	OGG	AGA	AAG	CAT	TTG	TTT	GTC	CAA	GAT	544
Cys	Glu	Pro	Cys	Ser	Glu	Arg	Arg	Lys	His	Leu	Phe	Val	Gln	Asp	
				170					175					180	
OCG	CAG	ACG	TGT	AAA	TGT	TOC	TGC	AAA	AAC	ACA	GAC	TOG	OGT	TGC	589
Pro	Gln	Thr	Cys	Lys	Cys	Ser	Cys	Lys	Asn	Thr	Asp	Ser	Arg	Cys	
				185					190					195	
AAG	GCG	AGG	CAG	CTT	GAG	TTA	AAC	GAA	OGT	ACT	TGC	AGA	TGT	GAC	634
Lys	Ala	Arg	Gln	Leu	Glu	Leu	Asn	Glu	Arg	Thr	Cys	Arg	Cys	Asp	
				200					205					210	
AAG	CCA	AGG	CGG	TGA											649
Lys	Pro	Arg	Arg												

FIG.6B

ATG CTG GCC ATG AAG CTG TTC ACT TGC TTC TTG CAG GTC CTA GCT	45
Met Leu Ala Met Lys Leu Phe Thr Cys Phe Leu Gln Val Leu Ala	
5 10 15	
GGG TTG GCT GTG CAC TOC CAG GGG GOC CTG TCT GCT GGG AAC AAC	90
Gly Leu Ala Val His Ser Gln Gly Ala Leu Ser Ala Gly Asn Asn	
20 25 30	
TCA ACA GAA ATG GAA GTG GTG OCT TTC AAT GAA GTG TGG GGC GGC	135
Ser Thr Glu Met Glu Val Val Pro Phe Asn Glu Val Trp Gly Arg	
35 40 45	
AGC TAC TGC CGG CCA ATG GAG AAG CTG GTG TAC ATT GCA GAT GAA	180
Ser Tyr Cys Arg Pro Met Glu Lys Leu Val Tyr Ile Ala Asp Glu	
50 55 60	
CAC OCT AAT GAA GTG TCT CAT ATA TTC AGT CCG TCA TGT GTC CTT	225
His Pro Asn Glu Val Ser His Ile Phe Ser Pro Ser Cys Val Leu	
65 70 75	
CTG AGT CCG TGT AGT GGC TGC TGT GGT GAC GAG GGT CTG CAC TGT	270
Leu Ser Arg Cys Ser Gly Cys Cys Gly Asp Glu Gly Leu His Cys	
80 85 90	
GTG GCG CTA AAG ACA GCC AAC ATC ACT ATG CAG ATC TTA AAG ATT	315
Val Ala Leu Lys Thr Ala Asn Ile Thr Met Gln Ile Leu Lys Ile	
95 100 105	
CCC CCC AAT CCG GAT CCA CAT TOC TAC GTG GAG ATG ACA TTC TCT	360
Pro Pro Asn Arg Asp Pro His Ser Tyr Val Glu Met Thr Phe Ser	
110 115 120	
CAG GAT GTA CTC TGC GAA TGC AGG OCT ATT CTG GAG ACG ACA AAG	405
Gln Asp Val Leu Cys Glu Cys Arg Pro Ile Leu Glu Thr Thr Lys	
125 130 135	
GCA GAA AGG TAA	417
Ala Glu Arg	

FIG.7

ATG CTG GGC ATG AAG CTG TTC ACT TGC TTC TTG CAG GTC CTA GCT	45
Met Leu Ala Met Lys Leu Phe Thr Cys Phe Leu Gln Val Leu Ala	
5 10 15	
GGG TTG GCT GTG CAC TOC CAG GGG GOC CTG TCT GCT GGG AAC AAC	90
Gly Leu Ala Val His Ser Gln Gly Ala Leu Ser Ala Gly Asn Asn	
20 25 30	
TCA ACA GAA ATG GAA GTG GTG OCT TTC AAT GAA GTG TGG GGC GGC	135
Ser Thr Glu Met Glu Val Val Pro Phe Asn Glu Val Trp Gly Arg	
35 40 45	
AGC TAC TGC OGG OCA ATG GAG AAG CTG GTG TAC ATT GCA GAT GAA	180
Ser Tyr Cys Arg Pro Met Glu Lys Leu Val Tyr Ile Ala Asp Glu	
50 55 60	
CAC OCT AAT GAA GTG TCT CAT ATA TTC AGT OCG TCA TGT GTC CTT	225
His Pro Asn Glu Val Ser His Ile Phe Ser Pro Ser Cys Val Leu	
65 70 75	
CTG AGT OGC TGT AGT GGC TGC TGT GGT GAC GAG GGT CTG CAC TGT	270
Leu Ser Arg Cys Ser Gly Cys Cys Gly Asp Glu Gly Leu His Cys	
80 85 90	
GTG GOG CTA AAG ACA GOC AAC ATC ACT ATG CAG ATC TTA AAG ATT	315
Val Ala Leu Lys Thr Ala Asn Ile Thr Met Gln Ile Leu Lys Ile	
95 100 105	
CCC CCC AAT OGG GAT OCA CAT TOC TAC GTG GAG ATG ACA TTC TCT	360
Pro Pro Asn Arg Asp Pro His Ser Tyr Val Glu Met Thr Phe Ser	
110 115 120	
CAG GAT GTA CTC TGC GAA TGC AGG OCT ATT CTG GAG ACG ACA AAG	405
Gln Asp Val Leu Cys Glu Cys Arg Pro Ile Leu Glu Thr Thr Lys	
125 130 135	
GCA GAA AGG AGG AAA ACC AAG GGG AAG AGG AAG CAA AGC AAA ACC	450
Ala Glu Arg Arg Lys Thr Lys Gly Lys Arg Lys Gln Ser Lys Thr	
140 145 150	
OCA CAG ACT GAG GAA CCC CAC CTG TGA	477
Pro Gln Thr Glu Glu Pro His Leu	
155	

FIG. 8

ATG CCG GTC ATG AGG CTG TTC OCT TGC TTC CTG CAG CTC CTG GGC	45
Met Pro Val Met Arg Leu Phe Pro Cys Phe Leu Gln Leu Leu Ala	
5 10 15	
GGG CTG GCG CTG OCT GCT GTG CCC CCC CAG CAG TGG GGC TTG TCT	90
Gly Leu Ala Leu Pro Ala Val Pro Pro Gln Gln Trp Ala Leu Ser	
20 25 30	
GCT GGG AAC GGC TCG TCA GAG GTG GAA GTG GTA CCC TTC CAG GAA	135
Ala Gly Asn Gly Ser Ser Glu Val Glu Val Val Pro Phe Gln Glu	
35 40 45	
GTG TGG GGC CCG AGC TAC TGC CCG GCG CTG GAG AGG CTG GTG GAC	180
Val Trp Gly Arg Ser Tyr Cys Arg Ala Leu Glu Arg Leu Val Asp	
50 55 60	
GTC GTG TOC GAG TAC CCC AGC GAG GTG GAG CAC ATG TTC AGC CCA	225
Val Val Ser Glu Tyr Pro Ser Glu Val Glu His Met Phe Ser Pro	
65 70 75	
TOC TGT GTC TOC CTG CTG CCG TGC ACC GGC TGC TGC GGC GAT GAG	270
Ser Cys Val Ser Leu Leu Arg Cys Thr Gly Cys Cys Gly Asp Glu	
80 85 90	
AAT CTG CAC TGT GTG CCG GTG GAG ACG GGC AAT GTC ACC ATG CAG	315
Asn Leu His Cys Val Pro Val Glu Thr Ala Asn Val Thr Met Gln	
95 100 105	
CTC CTA AAG ATC CGT TCT GGG GAC CCG CCC TOC TAC GTG GAG CTG	360
Leu Leu Lys Ile Arg Ser Gly Asp Arg Pro Ser Tyr Val Glu Leu	
110 115 120	
ACG TTC TCT CAG CAC GTT CCG TGC GAA TGC CCG OCT CTG CCG GAG	405
Thr Phe Ser Gln His Val Arg Cys Glu Cys Arg Pro Leu Arg Glu	
125 130 135	
AAG ATG AAG CCG GAA AGG AGG AGA CCC AAG GGC AGG GGG AAG AGG	450
Lys Met Lys Pro Glu Arg Arg Arg Pro Lys Gly Arg Gly Lys Arg	
140 145 150	
AGG AGA GAG AAG TAG	465
Arg Arg Glu Lys	

FIG. 9



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number

EP 92 30 2750

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	EP-A-0 399 816 (MERCK)	1-17	C07K15/06
Y	* the whole document *	18-20	C12N15/19
	---		C07K3/28
A	WO-A-9 102 058 (CALIFORNIA BIOTECHNOLOGY)	1-20	C12N5/10
	* the whole document *		A61K37/36
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A	WO-A-9 013 649 (GENENTECH)	1-20	
	* the whole document *		

A	EP-A-0 370 989 (MONSANTO)	1-20	
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P,A	EP-A-0 476 983 (MERCK)	1-20	
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P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 88, October 1991, WASHINGTON US pages 9267 - 9271; D. MAGLIONE ET AL.: 'Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor'	1-17	
			TECHNICAL FIELDS SEARCHED (Int. Cl.5)
P,Y	* the whole document *	18-20	C07K C12N A61K

The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 08 JULY 1992	Examiner MOLINA GALAN E.
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons</p> <p>& : member of the same patent family, corresponding document</p>			

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